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1 st International Conference
Strategies toward Green Deal Implementation

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Preface

The 1st International Conference on Strategies toward Green Deal Implementation – Water and Raw Materials (ICGreenDeal2020) was organised by the Division of Biogenic Raw Materials of the Mineral and Energy Economy Research Institute, Polish Academy of Sciences, on December 14–16, 2020. Due to the COVID-19 pandemic the conference was organised as the virtual event (online).

The conference turned out to be a great success for the organizers – almost 500 Participants attended the 1st edition of this conference. The 14 thematic sessions during this 3-days online conference included oral and poster presentations of 150 Authors. The following thematic sessions were the most popular among the participants:

- green deal strategies,
- cooperation for climate,
- sustainable management of water and raw materials,
- sustainable developments of regions,
- water and sewage in circular economy,
- raw materials for health care,
- bioeconomy,
- raw materials and waste,
- innovative materials for sustainable future,
- critical raw materials in green deal – phosphorus case of study,
- green strategies for waste and energy,
- actions for climate and circular economy,
- water – waste – energy in green deal.

The purpose of the conference was to present the issue of climate change and ways to prevent it through innovative solutions that could be implemented under the Green Deal Strategies, which have been developing dynamically in recent years in various regions of the world, such as North America, Europe or Asia. I hope that the exchange of knowledge and experiences during the conference have helped the Participants to get acquainted with the broad area of the strategies for implementing the Green Deal, with particular emphasis on water and raw materials.

The Monograph "Strategies toward Green Deal Implementation – Water and Raw Materials" includes the selected papers that have been presented during the 1st edition of International Conference on Strategies toward Green Deal Implementation – Water and Raw Materials.

I would like to thank all Authors and Reviewers for their valuable work in the preparation of papers and their reviews. Together, we act to save the Planet!

D.Sc. Marzena Smol
ICGreenDeal2020 Chair

*Michał PREISNER**

Wastewater treatment plants as an enabler of industrial symbiosis

Abstract: The study presents the importance of wastewater treatment plants (WWTPs) in the development of industrial symbiosis (IS) based on selected case studies. Industrial and municipal effluents are currently considered more often as a source of valuable resources rather than the a method for discharge of pollutants as it was in the previous decades. Nutrients, organics, cellulose, trace metals and energy can be recovered using various technologies at WWTPs. IS can be developed in municipal WWTPs by increasing the range of collected wastewater to not only urban effluents but also wastewater from some industries with a high content of valuable raw materials. Diary, food and beverage production are among the most interesting sources of materials that can be used in the IS. Recovered biogenic materials such as nitrogen and phosphorus are used for fertilizers production along with organic matter from digested sewage sludge. They can be applied in agriculture and improve the crop yields which further on are sold to the dairy and food and beverage producers. The study consists of five IS case studies analysis: in Kalundborg, Denmark, Forssa Region and Kemi-Tornio Region in Finland, Tymbark, Poland and Manresa in Catalonia, Spain. All of the analyzed case studies include an important aspect of WWTPs impact on the IS and present an approach to understand the potential enablers and barriers in terms of WWTP involvement in IS development and its transferability to other regions.

Keywords: industrial symbiosis, wastewater treatment plants, WWTP, nutrients, organics, agriculture, fertilizers, biogas, digestate

1. Introduction

Industrial symbiosis (IS) investigates the relationships between the industrial ecosystems and the environment [1]. IS development is based on involving separate industries in one collective approach aimed at obtaining competitive advantages deriving from the sharing of materials, energy, water and/or by-products [2]. IS is a subfield of industrial ecology (IE) that focuses on the

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study of industrial networks in which resources are shared and valorized [3, 4]. Moreover, IE with its tools can assist in the transition to circular economy (CE) and can be considered as the origin of this concept [5]. The authors of different studies state that the regional economy and economic geography should be the starting point for the development of IS [6]. This idea is in line with the current bioeconomy assumptions and reveals that regional IS can be initiated in the water and wastewater sector facilities such as wastewater treatment plants (WWTPs) [7]. Due to significant and important material and energy flows WWTPs which are present almost in every region around the globe have a high potential to become resource and energy recovery facilities (RERFs). The present study consists of an approach to extend this point of view to include the aspects of WWTPs as the enablers of regional IS systems by analyzing examples of successful cases of IS.

The idea of IS is not a foreign concept for industries. For decades, companies and industries have worked together, joining forces to operate more efficiently and reduce production costs [8]. Similarly to IE, the circular economy (CE) considers the industrial system as part of a larger ecosystem where the material and energy flows need to be optimized to limit the extraction of natural resources and reduce the impact on the environment, thus moving towards a more circular system [9]. The relationship between IE, CE and IS concepts and the place of WWTPs is present in Figure 1.

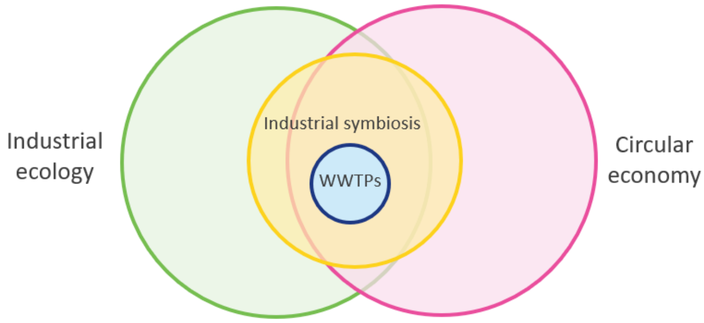

Figure 1.
Place of WWTPs in the relationship between IE, CE and IS concepts (own Figure, based on [3, 4, 10])

IE and CE have common goals and tools for their implementation [11]. One of them is IS which covers a large part of both mentioned above concepts. However, some aspects are not included in those ideas, such as waste-to-energy methods which are rather avoided in the CE framework [12]. Nevertheless, WWTPs are placed just in the middle of those 3 concepts due to a wide range of possible applications and material and energy flows involved.

WWTPs involvement in IS development is present among others in chemical industrial parks which were the first cases of IS implementation. With the industrial eco-parks development municipalities became an important stakeholder of the regional IS [13]. Due to the generation of municipal wastewater and waste, urban areas can provide a significant amount of resources and byproducts that can be further used by the industry [14]. Due to the constant reduction of greenhouse gases (GHGs) emission according to the Green Deal framework and increasing production of green and renewable energy, many IS cases included WWTPs as the main object for resources and energy recovery [15]. Especially the biogas production out of wastewater and its sludge gains importance while the current technological advance makes energy recovery from biogas easy and economically feasible [16]. Moreover, following the newest European Union's (EU) regulations, WWTPs will even extend their significance due to obligatory phosphorus (P) recovery from municipal sewage sludge in some Member States what can stimulate the creation of new IS initiatives [17]. As P was indicated by the European Commission (EC) as a critical raw material, its recovery from wastewater seems to be the most reasonable and sustainable method of providing an alternative source of this key element needed in so important sectors, as the food production industry [18].

Therefore, valuable resources, energy and water are among the main streams that have a high recovery potential in WWTPs (Figure 2).

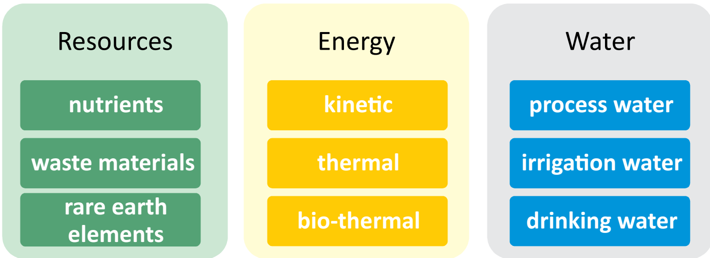

Figure 2.
Resources and energy flows in WWTPs (own Figure, based on [19])

Nutrients present in wastewater such as nitrogen (N), P and potassium (K) can be reused by the fertilizer industry [20]. Waste materials, including organics can be recovered by local farmers [21]. Wastewater consists of an amount of trace metals which can be valuable rare earth elements used in the electronic and chemical industry.

Different 3 types of energy can be recovered from wastewater [19]. By using the wastewater flow, e.g. in the outlet of sewage, it is possible to recover kinetic energy by installing microturbines. Moreover, thermal energy from excessive heat from households and industry can be recovered in the sewage system by heat pumps [22]. Bio-thermal energy refers to biogas production from sewage sludge anaerobic digestion [23]. As the most important resource for the planet Earth is water, we should reuse water as process water irrigation water in agriculture and urban green areas and in some specific cases, wastewater effluent can be recycled by advanced treatment to potable water quality [24].

2. Materials and methods

A set of data related to the existing IS cases was collected in order to provide the necessary input for the present study. The data was gained from a total of 44 scientific papers published in international, high impact factor journals, 8 reports on IS and CE published as a result of international projects, 2 technical magazines describing the development of IS examples and 4 presentations published online. The collected data were screened for the most relevant aspects of WWTPs operation for the IS development and the type of cooperation involved in IS. From the identified examples 5 of them were chosen as the most representative cases to be analyzed in the study as shown in Table 1.

Table 1.
Analyzed IS cases with the importance of WWTPs for IS development and references

IS name and location	Type of cooperation	Importance of WWTP for IS development*	Reference
Kalundborg symbiosis, Denmark	Industrial ecosystem	+++	[9, 25, 26]
Forssa Region, Finalnd	Multidimensional regional symbiosis	++	[27, 28]
Kemi-Tornio Region, Finland and Sweden	Natural resources refining IS	+	[29–31]
Tymbark, Poland	Food and beverage based IS	+++	[32, 33]
Manresa en simbiosis, Catalonia, Spain	Multidimensional regional symbiosis	++	[34, 35]

* Moderate importance (+), high importance (++), critical importance (+++).

The selection of final IS cases was imposed by the relevance of WWTPs for the case initiation and development and to obtain a wide variety of IS examples from different countries and types of stakeholders involved. Furthermore, the case analysis was performed by investigating the following aspects: evidence of success (I) and difficulties encountered (II).

3. Results

The research methods were applied to investigate the following cases of IS. The results of quantitative data analysis (evidence of success) along with the identified difficulties encountered are provided in Table 3 after the description of IS examples studied.

3.1. Kalundborg symbiosis, Denmark

Kalundborg symbiosis is a model and the oldest example of IS according to the principle of proximity [36]. The Danish IS example was initialized in the 1960s when the energy and oil company Esso (currently Statoil) needed water for its refinery near Kalundborg and led the pipeline from the nearby Lake Tissø [36]. Practically, it was not a IS project than, but the pipeline was a public-private partnership (PPP) between Kalundborg municipality and the refining company and remains one of the key elements of Kalundborg symbiosis while companies still utilize surface water instead of the more valuable and scarce groundwater resource [37]. Furthermore, the energy company Asnæs Plant (currently Dong Energy) was connected in 1973 to the existing Statoil pipeline. This initiated a symbiosis by sharing resources and infrastructure by two entities. In the following years, more companies located in the Kalundborg area joined in a cooperation network by exchanging materials and energy.

As an example of material and energy exchange with the involvement of a WWTP, the plant belonging to a biopharmaceutical company and the largest producer of enzymes in the world (Novozymes), which recovers nutrients, enabling further fertilizer production and excessive heat recovery from industrial wastewater. Their WWTP accepts also wastewater from another IS participant – Novo Nordisk (the largest insulin in the world), which is used for biogas production while the digestate is used by local farmers to improve agricultural production [36]. This example of WWTP involvement in the IS gives wide opportunities for further transfer of this good practice to other industrial parks with WWTP operating.

Moreover, Kalundborg has its biogas and natural gas desulphurization installation sells the captured sulfur to Gyproc company for the production of gypsum which can be easily used in the construction industry. Residual heat is sent to the district heating system of the Kalundborg municipality while the fly ash from Oested (residual from coal and biomass burning) is given to the local cement producer to become a raw material for new buildings in the eco-industrial park [36].

3.2. Forssa Region, Finland

The IS in Forssa, Finland, is a perfect example of regional IS with an important role of wastewater aspect involved. In general, the symbiosis is based on the materials and waste exchange of 3 stakeholders: a meat company – HK Scan Finland, glass wool insulation producer – Saint Gobain Isover and Envor Group Oy – the biogas plant operator [27]. The symbiosis generates secondary materials such as fertilizers, biogas and biofuels including actors from 3 different sectors food production, construction and biogas production.

HK Scan Finland has its factories located in Forssa Finland and all of its bio-based waste and by-products (e.g. meat offal) are transferred to Envor Group as input for biogas production. A family-owned Envor Group Oy produces biogas and biofuels from bio-waste and biosludge from wastewater treatment. This allows Saint Gobain Isover to substitute propane gas with biogas transferred from Envor Group for the glass wool production process [28].

Because of close connections between Envor Group and Saint Gobain Isover, the companies decide to build a pipeline between the two companies. The pipeline enables Saint Gobain Isover to use biogas as a substitute for propane gas, which is an opportunity to achieve environmental benefits regarding CO_2 emissions. Furthermore, the digestate from the biogas production can be used for grain production by local farmers.

3.3. Kemi-Tornio Region, Finland

The IS in the Kemi-Tornio Region is based on wide-range cooperation between industrial entities located at the north Baltic Sea shore, close to the Finish-Swedish border. It is the world's northernmost center of bio, mining and metal industry with 1700 tons of by-products and residues (excluding waste rock) exchanged bringing approx. 700 million € annually and responsible for 7–8% of the total export value of Finland [31]. The IS system consists of 5 biorefineries, 32 sawmills, 16 mines, 5 metal refineries, aluminum smelters, 1 liquid natural gas (LNG) refinery, 2 chemical plants. The biggest companies among IS partners are Stora Enso (pulp mill), Metsä Group (forestry industry), Outokumpu (stainless steel industry), Manga LNG terminal and waste management companies. It is planned that also mining industry will join the IS activities and the symbiosis will develop gradually [29].

Due to the location of the IS system at the Bothnian Bay shore, there is a high risk of water pollution by industrial wastewater [38, 39]. While the IS consists of a pulp mill and other facilities with a potentially high content of biogenic substances in the effluents the risk of eutrophication is high [40]. Moreover, when taking into account the actual quality of water in terms of its trophic state it is clear that the effluent recipient water body is sensitive to P loads [41]. To limit the risk of eutrophication the wastewater treatment systems reduce biogenic substance loads and allow to recover pulp sludge for further processing by IS partners and promote sustainable bioenergy resources by biogas production at the industrial WWTPs [42].

3.4. Tymbark, Poland

Tymbark company is the largest producer of fruit juice-based beverages in Poland and one of the largest in central-eastern Europe. The actual on-site juice production is mainly based on apples and seasonally from blackcurrant and black chokeberry (Aronia). The fruit pomace produced as a byproduct of the juice production process is bought from local farmers to be an additional fed for the biogas production [32].

The plant in Tymbark, located in southern Poland (Małopolska Region) operates an innovative combined heat and power (CHP) system, based on the aggregate cogeneration with an electricity capacity of 1.0 MWe, fed with biogas and high-methane natural gas. The plant owns a WWTP equipped with a biogas reactor in the anaerobic zone in which from the treated wastewater biogas is produced resulting in 2350 MWh of bioenergy achieved per year [33]. The CHP system is used for chilling water production in summer and to cover demand for heat energy and processing steam in winter [32]. Furthermore, due to the high capacity of the WWTP, the Tymbark plant accepts external effluent from the Tymbark municipality (6500 inhabitants) and a local dairy plant OSM Limanowa.

3.5. Manresa en simbiosi, Catalonia, Spain

The implementation of the pioneer IS project in Manresa (Catalonia, Spain) was aimed at achieving the highest resources efficiency and to initialize the synergies between companies and entities such as Bufalvent Business Association, Diputación de Barcelona, Manresa Technology Center (Eurecat), Waste Agency of Catalonia, Manresa City Council and Bages Waste Treatment Consortium [34].

The IS system includes a WWTP with a high-capacity biogas reactor to which organic residues are transported from the nearby industries. The largest source of organic matter for the biogas production is a slaughterhouse owned by Mafrica company, which transfers approx. 3000 tons of meat offal per year to the biogas plant at the WWTP [35]. Other sources of needed resources for biogas production are municipal effluents from the urban areas. The produced biogas is used

Table 2.
The IS cases analysis

IS case study	Evidence of success	Difficulties encountered
Kalundborg symbiosis, Denmark	• Reduction of 635 000 tons of CO_2 emissions, • 3.6 million m^3 of saved water, • 100 GWh of energy, • 87 000 tons of different materials, • 25 different resource streams exchanged, • over 5000 workplaces, • 24 million € of economic benefits, • 14 million € of socio-economic benefits.	• Public-private partnership initiation, • environmental regulations in terms of by-products and waste definitions, • lack of experience during the IS initiation.
Forssa Region, Finland	• Emissions reduction of CO_2 by 19,600 tons in 2010–2018, • approx. 200 000 € economic benefits total.	• Maintaining steady methane content and ensuring constant supply during power failures. • Constant supply has improved by adding more pressurized storage capacity beside Saint Gobain's pipeline.
Kemi-Tornio Region, Finland	Several waste streams have been recovered e.g.: • water purification precipitate: 20,000 tons per year, • biosludge: 12,000 tons per year, • slaked lime: 30,000 tons per year, • burnt lime: 2,000 tons per year, The value of IS and environmental business in the area is estimated to be at 200 million € annually.	• In some cases, existing regulations have caused legal problems, • Funding, lack of financial support, • Lack of expertise/skills of existing employees within firms involved, • High imbalance of power between partners (sizes of companies), • Different organizational cultures within firms can difficult cooperation.
Tymbark, Poland	• Production of 300,000 m^3 of biogas per year (approx. 2350 MWh), resulting in 30% savings on heating purposes, • Significant fruit pomace waste reduction.	• Dependence of the Tymbark's WWTP from the local farmers in terms of annual fruit pomace production, • Fluctuations in the flow in the buffer tank.
Manresa en simbiosi, Catalonia, Spain	Reductions in waste generation: • 11,000 tons of municipal waste, • 256 tons less are sent to landfill, • 11 tons of raw materials saved, • 12 GWh of heat saved, • 7 GWh of electricity generated, • 1,200,000 € potential savings in energy generation, • 135,000 € potential savings in waste management.	• Funding, lack of financial resources, • Lack of expertise/skills of existing employees with-in firms involved.

for heat energy production and is sent to the district heating system and as electric energy to the local steel mill. The residues after the biogas production (digestate) is transferred for composting and can be reused for green areas maintenance [43].

4. Discussion

The results of the present study correspond with the work of other authors which have made a significant contribution to the IS development scientific knowledge by showing the enablers and barriers to obtaining and maintaining IS [9, 44–46]. Among the most often mentioned enablers in the most cited publication, we can find incentives such as policy instruments, economic benefits, geographical proximity, technological know-how, easy stakeholder participation, etc. [47–50]. All of the above enablers correspond with the inclusion of WWTP in the IS systems. Modern and advanced WWTPs are more often considered as RERF rather than just water environmental protection facilities [51]. This allows initiating new IS systems or develop the existing ones according to the CE concept by closing the loop of resources [52].

Unfortunately, like the enablers also barriers to initiate or develop IS are similar in terms of WWTP impact on IS. The main barriers are: not sufficiently developed waste and by-product market, missing regional incentives by local/regional governments, poor governmental financial support, lack of clear waste definitions and a long return rate of necessary investment [53–55].

However, WWTPs with their possibility to recover nutrients (such as valuable P) and energy from biogas give a major advantage over other IS stakeholders. Moreover, WWTPs exists almost in every region (even local and rural areas) and have similar operating methods what simplifies their inclusion in IS with partners from different industrial sectors [56].

On the other hand, the shortcomings and critiques about WWTP as an IS element should be also considered. While, there are no serious concerns about nutrient recovery, which is a sustainable method of obtaining biogenic matter and an alternative to raw material extraction, there are doubts about the sustainability of biogas use for heating purposes within the waste-to-energy concept which is not the first option in the CE framework [57]. However, due to the lack of affordable environmental technology for providing sustainable district heating or cooling, biogas has a much lower environmental footprint than biomass burning or fossil fuel combustion [58]. Therefore, biogas obtained in WWTP, with potential excessive bio-based waste input and remote biorefineries remains one of the most sustainable solutions at the current state of the art.

5. Conclusions

The study presented the aspect of WWTPs impact on IS development by using case studies from various regions. The results allowed to state that WWTPs have a significant influence on the dynamics and direction of IS systems creation, with major contribution obtained from WWTPs operation. Possible resource and energy recovery gives wide opportunities to use WWTP capacities to increase nutrient recovery and biogas production. The analyzed case study from Tymbark, Poland proved that WWTPs are able to extend the stakeholder list by local farmers especially when the fruit and beverage industry is involved in the IS. Moreover, meat production can limit its high environmental footprint by transferring the slaughterhouse offal to a biogas plant, which produces sustainable bioenergy and digestate with a high content of organic matter that can be reused by local farmers (as in Manresa, Spain and Forssa, Finland). Furthermore, pulp and biomaterials plants can limit their negative impact on the water environment by reducing loads of pollutants with high eutrophication potential as the facilities in the Kemi-Tornio Region in Finland.

Due to the conducted analysis of potential enablers and barriers of IS development with the WWTPs participation the paper presented traps that should be avoided when developing such system giving valuable tips for policymakers and future initiators of IS systems.

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State of the art of heavy metals removal from wastewater including strategies for the management of generated wastes

Abstract: Heavy metals are dangerous pollutants now commonly present in surface water. Because of the increasing problem with pollution, there is a need to develop the technologies of their removal from wastewater, but including the strategies of the management of wastes generated during the process. A short review on the state of the art in this area is presented in the paper. Physicochemical, chemical, electrochemical methods are presented, as well as ion exchange and membrane filtration. Also biological methods are shortly described. Electrochemical methods are considered to not generate waste products, they also allow to obtain homogenous precipitates of metal hydroxides, and no chemicals are required. Taking into consideration waste products management they are optimal, but high capital and maintenance costs may be required. Such processes as ion exchange and filtration methods produce waste liquids rich in metals and small in volume. They need further management. Adsorption methods are effective but the adsorption capacity of adsorbents (both biosorbents and other types) makes it suitable for the removal of metals from rather diluted solutions. Regeneration of adsorbents is possible, but it is connected with losses of the material and changes in the surface chemical structure. In individual cases, the method must be chosen based on individual analysis.

Keywords: industrial wastewater, heavy metals, waste management

1. Introduction

Heavy metals are important pollutants of many industrial wastewater. They are defined as metals whose density exceeds 5 g per cm^3. Among heavy metals, we can distinguish the ones called trace elements: cobalt, copper, zinc, and the ones which pose a serious threat for the environment: lead, cadmium or mercury [1]. The latter in particular pose a serious risk for living

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organisms. But all heavy metals can be dangerous because of their solubility in water (it depends on pH and other environmental conditions), toxicity, tendency to accumulate in cells and tissues. As a result, e.g. nervous system disorders, cancer, or damage of the organs and cells can occur. The toxic effects of heavy metals depend among others on the dose of the pollutant to which living organisms are exposed, on the duration and route of the exposure, chemical species, but also age and nutritional status of exposed individuals [2]. During wastewater treatment, part of the metals is cumulated in sludge or precipitates, but part of these pollutants is discharged to the surface waters. As a result pollution of the water environment is the important problem of today's world. It can be seen e.g. based on the data given in Figure 1. The data presented in the Figure were collected by Zhou et al. [3] based on a literature review for the period from 1972 to 2017, from all over the world. The data indicate that over the mentioned time the pollution of water bodies significantly increased, moreover it has changed from single metal pollution to pollution by various metals [3].

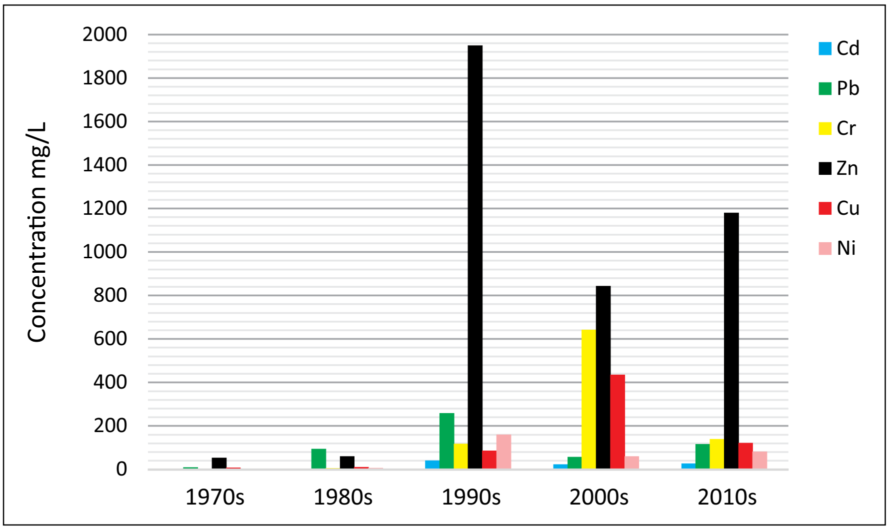

Figure 1.
Changes in mean individual heavy metal concentration in river and lakes worldwide from 1972 to 2017 based on the data collected by Zhou et al. [3]

The data should be of course considered as a tendency, not specific values. The problem is emerging, and because of this, there is a special need to develop methods of heavy metals removal from wastewater, to protect the environment from these toxic pollutants. What is equally important, but less frequently discussed in the literature, are strategies for the management of wastes generated during the removal of heavy metals from wastewater. Removal of heavy metals and management of the waste products containing these micropollutants fits well in the European Green Deal which assumes the achievement of a toxic-free environment [4]. There is a need to develop the treatment methods including management strategies that are not only economically viable and technologically effective but also generate as few final hazardous wastes as possible.

The present study aims to present state of the art in heavy metals removal from wastewater including management of the generated waste products. Technologies of heavy metals removal from wastewater were described. The efficiency of the methods as well as cost-effectiveness were first of all discussed. After that, the strategies of waste management generated during various treatment methods were described as the important aspect of the practical application of the methods.

2. Technologies of heavy metal removal from wastewater

Methods of heavy metals removal from wastewater can be divided as: physicochemical, chemical, electrochemical, ion exchange, membrane filtration, and biological [1].

2.1. Physicochemical and chemical methods

They include among others chemical precipitation (including separation of precipitates), coagulation (including separation of the solids), and adsorption. The principle of precipitation is to remove heavy metals from the water phase through the formation of the salts (e.g. sulfates, sulfides, phosphate, carbonates) or hydroxides of heavy metals. In this form, metals can be easily separated from the liquid phase. The efficiency of the process depends on the environmental conditions such as pH, temperature, initial concentration of heavy metal ions, presence of other anions or cations [1, 5]. Coagulation and flocculation also allow for the removal of heavy metals and precipitate them in form of sludge. The processes mentioned above are economically viable, especially if the concentration of heavy metals in solution, and as result, the volume of precipitate/sediment are high [1]. High volumes of the precipitates containing heavy metals can be considered as cons of the method. The advantage of the process is its simplicity and low capital costs, however, it is not metal selective, and maintenance of the process is rather high because of high chemical consumption [6].

During adsorption heavy metals are adsorbed onto the surface of various adsorbents, including e.g. powdered or granular activated carbon. As a result ions of the metal are effectively removed from the water solution and cumulated on the adsorbent. This method is effective in the case of rather low concentrations of heavy metals because of the costs. Some research on alternative adsorbents has been performed including waste iron, fly ash, etc. [1]. The advantage of the process is its simplicity and effectiveness, but high costs and difficult regeneration of adsorbent can be noted as disadvantages [6]. The sorption capacity of inorganic adsorbents is usually in the range of a few to several mg per g of adsorbent [6].

Recently graphene oxide has been suggested as an effective adsorbent for heavy metal removal [7]. It can be applied e.g. in the form of epoxidized nanotubes. Adsorption efficiency of heavy metals was higher than 99% towards water solutions of Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, and Pb^{2+} of initial concentration in the range 60–6000 ppm [7]. Also, other nanomaterials (e.g. biopolymers, zeolites) can be used for heavy metals removal. They have high reactivity, large surface contact, and good disposal capability. Nanofibers are also frequently used as components of membranes [8].

Other innovative adsorbents are hydrogels [9]. Hydrogels used in heavy metal ions removal can contain various functional groups, e.g. carboxylic acids, amines, hydroxyls, or sulphonic acids. They for example can be used as complexing agents for the removal of heavy metal ions [9]. As in the case of other adsorption methods pH plays important role in the effectiveness of hydrogels adsorption of heavy metals. The removal efficiency of heavy metals over 90% can be achieved by using hydrogels.

2.2. Electrochemical methods

These methods use electricity to pass a current through an aqueous metal-bearing solution in which the cathode and anode are submerged. As a result, heavy metals are precipitated in a weak acidic or neutralized catholyte as hydroxides [1]. This method allows to obtain homogenous precipitates of metal hydroxides which makes it easier to manage the waste material. The advantage of the method is that no chemicals are required, however high capital costs are necessary [6]. The efficiency of the process can be very high, even 99.9% [10].

2.3. Ion exchange

Ion exchange also removes heavy metals from the solution and cumulates it in the solid phase (ion exchanger). Because of costs, this method is mainly used in water treatment, not wastewater. If wastewater is treated it is applicable for the removal of rather low concentrations of heavy metals. As ion exchangers, synthetic organic resins are usually applied [1]. Contrary to adsorption methods the ion exchangers can be regenerated, and as a result, the solution containing high concentrations of heavy metals occurs. It can be metal selective [6].

2.4. Membrane filtration

During membrane filtration, heavy metals are retained on membranes of various pore sizes. Generally, the membrane acts as a barrier that inhibits the passage of individual constituents while others can pass through this barrier [10]. Treated and collected streams are called permeate; rejected constituent is called retentate. The method includes ultrafiltration, nanofiltration, and reverse osmosis. Ultrafiltration membranes with pore sizes between 0.01 µm and 100 µm are considered as not especially effective in heavy metals removal, but some methods using this type of membrane have been used, e.g. at low transmembrane pressure or with embedding adsorbents onto membrane matrix. Nanofiltration membranes naturally have a loose selective thin film structure and small pore sizes which are sufficient for metal removal. Reverse osmosis membranes are denser than the nanofiltration ones and do not have definite pores, they work in opposite direction (pressure is exerted to force water molecules to move against the concentration gradient) [10]. The effectiveness of the membrane filtration methods reaches usually more than 90% of heavy metal removal and depends on heavy metal ion, solute concentration, pressure, water flux rate [1]. By using the most effective reverse osmosis methods up to 99% heavy metals rejection can be achieved [10]. The advantage of the process is also that no high space is required to maintain it [6]. As the disadvantages high costs of investment and maintenance are indicated, but lately these decreased a lot, by the development of new types of membranes.

Membrane processes can be applied also as hybrid ones, e.g. combining adsorption, membrane separation, and flotation. It allows selective removal of heavy metals from water or wastewater. The process can be divided into three steps: adsorption by bonding agent, separation of bonding agent from wastewater by filtration, e.g. microfiltration, and regeneration of bonding agent [8].

As a result concentrated liquid waste is generated, however low solid waste quantities are generated. Membrane filtration can be supported by applying an electric potential to support passing the ionized species through an ion-exchange membrane. This process is called electrodialysis [1].

2.5. Biological methods

Biological methods include the removal of heavy metals by using activated sludge or attached biomass. These methods are not effective in metal degradation, the metals are cumulated in the sludge and this phenomenon affects the further utilization of this waste material.

As a biological method also biosorption is classified. Various low-cost biological sorbents can be applied for the removal of heavy metals from wastewater, including biomass, algae, agricultural wastes, etc. [1, 4]. Biological adsorbents such as algal biosorbents allow for 90–99% removal of heavy metals under optimal conditions; use of microbial adsorption processes is less effective, with 70–75% of heavy metal removal efficiency, similarly to the use of biopolymer adsorbents [11]. The adsorption capacity of biowastes can reach even a few hundred mg per g, however usually it is not higher than several dozen mg per gram [6]. The biomass especially effective in heavy metals removal is Spirulina and Fucusceranoides, but also Pseudomonas or Spirogyra [6]. Technical parameters of the biosorption may vary a lot depending on the type of adsorbent used. Applied adsorption times are from 10 -20 min to several hours; pH can be in the wide range from less than 3 to even 8. The parameters must be chosen experimentally in individual cases [6].

3. Strategies of management of waste products generated during heavy metals removal from wastewater

For the above, all methods generate various waste products which require further processing. The waste products and strategies of their management are as follows.

3.1. Physicochemical and chemical methods

In the case of adsorption, various chemicals are used for the desorption of heavy metals from adsorbents.

The chemical used for this purpose depends on the kind of adsorbent. It can be by using specific solvents, diluted acids (the most frequently used are HCl or HNO_3) or organic acids, or by oxidizing chemical agents, also under subcritical or supercritical conditions [6]. It can be concluded that the efficiency of chemical methods is primarily affected by the solubility of heavy metals in chemical solvents. Recovery of desorbing agent is the desired step of the process [6]. Despite chemical regeneration also other methods can be used, e.g. thermal processes or biological recovery methods. Thermal processes can be applied e.g. in the case of activated carbon. This method is however expensive and can cause damage to the adsorbent [6]. Biological methods are cost-effective, but they require a long reaction time and are rather not applicable in the case of non-degradable compounds [6]. In the case of heavy metal removal practically only chemical methods of sorbents are applied. The efficiencies of the processes vary from a few percent to even 98%, e.g. by using NaOH (0.1 M) as eluent 90% of chromium were leached from metal oxides sorbent material, whereas by using NaCl (0.1 M) 59% of copper was eluted from spruce sawdust [12]. Recovery of zinc from activated sludge by using saponins reached 95%. The same biosurfactant used for the recovery of cadmium gave 51.2% removal efficiency [12]. In the case of heavy metals desorption process is pH-dependent [6]. The problem is however further management of leaching agent enriched in heavy metals desorbed from the adsorbent. Innovative adsorbents such as epoxidized graphene nanotubes can be regenerated e.g. with a diluted solution of acetic acid [7]. This is an economically effective regeneration method as acetic acid can be easily produced by bacteria. Such adsorbents as hydrogels are also considered as easily retrievable and reusable. Another advantage is that hydrogels can be biodegradable and biocompatible [9]. As the method of hydrogel regeneration is considered to expose this agent, after using it for heavy metal removal, to several desorption cycles in an acidic, basic solution or a salt, depending on the type of hydrogel and type of heavy metal. They are effective processes that allow for the recovery of more than 80% of the adsorbed metal ions [9].

3.2. Electrochemical methods

No reagents are necessary in the case of electrochemical methods. This method also does not normally generate any secondary by-products [10]. Because of this, taking into consideration waste management, they seem to be attractive alternative to the other methods. However, the problem is the required electric potential. It limits economic viability of this method. Another problem is that during the process the conductivity can be lowered and the electrical resistance will be increased, what decreases the efficiency [10].

3.3. Ion exchange

For recovery of heavy metals from anion exchange resins could be e.g. by using carbonic acid as a regeneration agent by dissolving supercritical CO_2 in water by high pressure. Based on the research work done by Silva and Brunner [13] this method can be suitable for Cu and Cd. The authors have stated that the parameter which had an important effect on recovery effectiveness was feed concentration, whereas temperature not affected the process significantly [13].

3.4. Membrane filtration

Waste products generated as a result of membrane are concentrated retentate. The retentate containing heavy metals which must be further managed, by using various methods, including e.g. precipitation (for heavy metals recovery) [14]. The process is effective but the waste stream is difficult to handle, however the volume of the solution is reduced (to about 10-20% of the initial volume, 20 – 85% of initial metal concentration) what makes it more suitable for further treatment because concentrated stream of small volume is easier for precipitation than other ones [14]. In some cases, precipitation of metals can be coupled with other processes, e.g. reduction. This is appropriate in the case of chromium. Hexavalent chromium can be reduced to trivalent one, and after that precipitation is performed [14].

In the case of hybrid processes including adsorption, membrane separation and flotation regeneration of bonding agent is also possible [6].

3.5. Biological methods

In the case of biosorbents desorbing medium which is used for the regeneration should primarily not damage the biosorbent (biomass). Various solutions can be used for biomass regeneration including e.g. acids (for example HCl), bases (for example NaOH), or chelating agents [8]. If biosorbents are used high efficiencies of heavy metal recoveries from sorbents can be achieved, e.g. by using 0.1 EDTA about 91% of lead was recovered from Spirulina maxima biomass. HCl allows for the removal of more than 90% of heavy metals ions [8]. The use of this acid simultaneously causes hydrolysis of the functional groups of the biomass; as a result, it can affect biosorption efficiency of the regenerated biosorbent [8]. Recovery of heavy metals from chitosan also gave satisfactory results. By using as eluting agents diluted mineral acids or organic acids up to 88% of chromium can be recovered [15]. The research work made by Vakili et al. [16] has indicated that acidic eluents are the most efficient in the desorption of chitosan-based adsorbents. They are also the most frequently used ones [16].

4. Conclusions

The review given above confirms that technologies of heavy metals removal which meet sustainable development rules should take into consideration not only efficiency removal and technological parameters of treatment method, but also management strategy of waste products management.

Electrochemical methods are considered to not generate waste products, they also allow to obtain homogenous precipitates of metal hydroxides, and no chemicals are required. Taking into consideration waste products management they are optimal, but high capital and maintenance costs may be required. Such processes as ion exchange and filtration methods produce waste liquids rich in metals and small in volume. They need further management. Adsorption methods are effective but the adsorption capacity of adsorbents (both biosorbents and other types) makes it suitable for the removal of metals from rather diluted solutions. Regeneration of adsorbents is possible, but it is connected with losses of the material and changes in the surface chemical structure. In individual cases, the method must be chosen based on individual analysis.

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Livestock building in organic farms. Existing constructive solutions in the Masovian voivodeship – a case study

Abstract: The paper presents results of direct investigations concerning livestock buildings and their modernization in selected organic farms of the east Masovian voivodeship. As a research area, the counties of Sokołów Podlaski and Węgrów were chosen. The livestock buildings in 14 farms were investigated. The investigations were carried out with use of on-site visit and talks with their owners. A research objective consisted in an attempt of finding connections between the organic animal breeding and building substance (livestock buildings). The study has shown that agricultural practices were more connected to changing the farming methods to organic ones than the materials used in livestock buildings. Materials which were used to erect the livestock buildings are i.a. full lime-sand brick, full ceramic brick and cellular concrete.

Keywords: livestock building, organic farm

1. Introduction

Increasing pollution of the natural environment enforced the European and worldwide governments to commence activities concerning human's health and life as well the natural environment and its protection. The agriculture which produces food is a field closely and directly depending on the nature. High quality food products cannot be produced in a polluted environment. The concept of the sustainable development – used to be called eco-development – assumes such management of natural resources which not only will not deteriorate them but also – as far as possible – will contribute to their development and improvement [1]. One of the means of

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agricultural activity originating from such worldview is undoubtedly the organic farming. At the beginning it was based on intuitive activities and the concept of action in accordance with the nature resulted from philosophical motivations. "*Organic farming is not a regress in the civilization development but it constitutes the most modern form of farming, based on the knowledge of natural laws*" [2].

The term "organic farming" has been acknowledged in the legislation of the European Union (EU) [3] as a synonym of the much older term "biological farming", originating from historical names of methods of the agricultural production, the methods which had been developed independently in various countries and contributed to the derivation of rules of the organic farming. The area of the organic farms in 2019 constituted 8.5% of the total agricultural area in the EU, of which only 3.5% in Poland [4]. As at 31.12.2020, there are 20274 organic producers registered in Poland, of which 18575 lead organic farming. In the Masovian voivodeship, it is 2661 and 2179, respectively. The area of organic farms in Poland is 509 291.27 ha, in the Masovian voivodeship – 41217.95 ha, what makes 8% of the total area [5].

The manufacturing technology of building materials allows application more and more economical and ecological materials as well as whole construction systems, used to erect building objects consuming much lower quantities of energy at the stage of construction and exploitation [6, 7]. A building becomes a part of the ecosystem and the building construction should be ecological [8–13]. According to Mikoś [14], the main objectives of the ecological building construction are: significant improvement of health potential of buildings, i.e. minimization of factors harmful to the health; significant reduction of environmental impact of buildings and their conglomerations, i.e. minimization of environmental pollution; minimization of energy consumption during erection and exploitation of buildings and objects, maximum use of renewal energy; shaping buildings and their conglomerations with maximum use of natural plants as a biological coating of the buildings. It is suggested by foreign authors as well [15, 16]. A necessary but insufficient condition for the systems acknowledged as ecological is a low energy consumption for exploitation of a building. An important case is a requirement of the low energy consumption in the whole cycle of the so-called "building life": from acquisition of raw materials, through manufacturing of products, construction and exploitation of the building, its maintenance (durability requirements), modernization and demolition to the re-use of these materials after the demolition. These processes should fulfill health requirements: it concerns the phases from the acquisition of raw materials to the recycling of the materials from the demolition [14].

The organic farming was addressed in many publications concerning agrotechnical measures, economy and profitability of production, market and quality of the organic products, animal breeding, possibilities of its development, environmental impact.

The issues of ecological building materials are known in the literature. It was the object of investigations of Mikoś [14], Sawicki [17], Janiak [18, 19], Pawlikowski [20], Racięcki [21], Stachowicz et al. [22, 23], Żurakowska [24], Kupiec-Hyła [25, 26], Kelm [27], Brzyski [28]. The publications todate concern each problem − organic farming, ecological building construction, ecological building materials − separately. However, there are no publications concerning these three problems as mutually merging and supplementing each other. Thus, the objective of this study is a synthetic presentation of all abovementioned aspects and their mutual influence.

In the years 2001−2004, some publications were featured which presented investigations on buildings in Polish organic farms [29−31]. In that time, the organic farms were unified in two societies and their number did not exceed 600 Poland in total. It was mainly a legally unsanctioned activity (basing on a voluntary following the rules developed by the organic farmers' societies). After the Polish access into the EU in 2004, legal foundations of the present status of organic farms commenced. In the next years, publications featured which concerned dwelling buildings localized in the certified organic farms in various regions of Poland [32−35]. Results of these investigations proved a slight influence of the organic character of farms on dwelling houses.

The authors of this study decided to verify the conclusions resulting from the publications from the early 2000's with relation to livestock buildings. In this aim, the livestock buildings in the selected areas of Poland were investigated from the point of view of application of ecological building materials. It was checked, whether and to what extent the organic character of farming affects transformations (reconstructions, renovations) of the livestock buildings.

2. Materials and methods

Hereby, the material presents a resumée of a part of the investigations concerning buildings localized in homesteads and dedicated for farmed animals – livestock buildings – in the selected part of Poland.

The research was conducted in the east part of the Masovian voivodeship, in the counties of Sokołów Podlaski and Węgrów. In the first selection 35 organic farms in the Sokołów county and 43 farms in the Węgrów county were taken into consideration(Figure 1). From the selected 78 farms in these two counties, the owners of 45 of them did not agree for the visit, thus finally 33 farms were visited, among them 14 have livestock buildings.

The methodology of the investigations consisted in an on-site visit comprising a description of the objects and collection of photographs, direct interview with buildings' owners, concerning the building substance (year of construction, dates of modernization, information on material and constructive solutions of foundations, walls, ceilings, rafter framing – all data which were unable to obtain during the visual inspection).

3. Results

Most of the farms (8) have an area 10–20 ha, three of them have under 10 ha, three – over 20 ha (25, 50 and 80 ha). The majority (72%) of the farms switched to the organic farming 10–12 years ago. Unfortunately, the animal breeding in organic farms is permitted in so-called "conventional mode", merely in two farms (14%) it was declared that the breeding bases on ecological methods. Most of the farms are specialized: 11 farms breed cattle, 2 – horses, one – pigs.

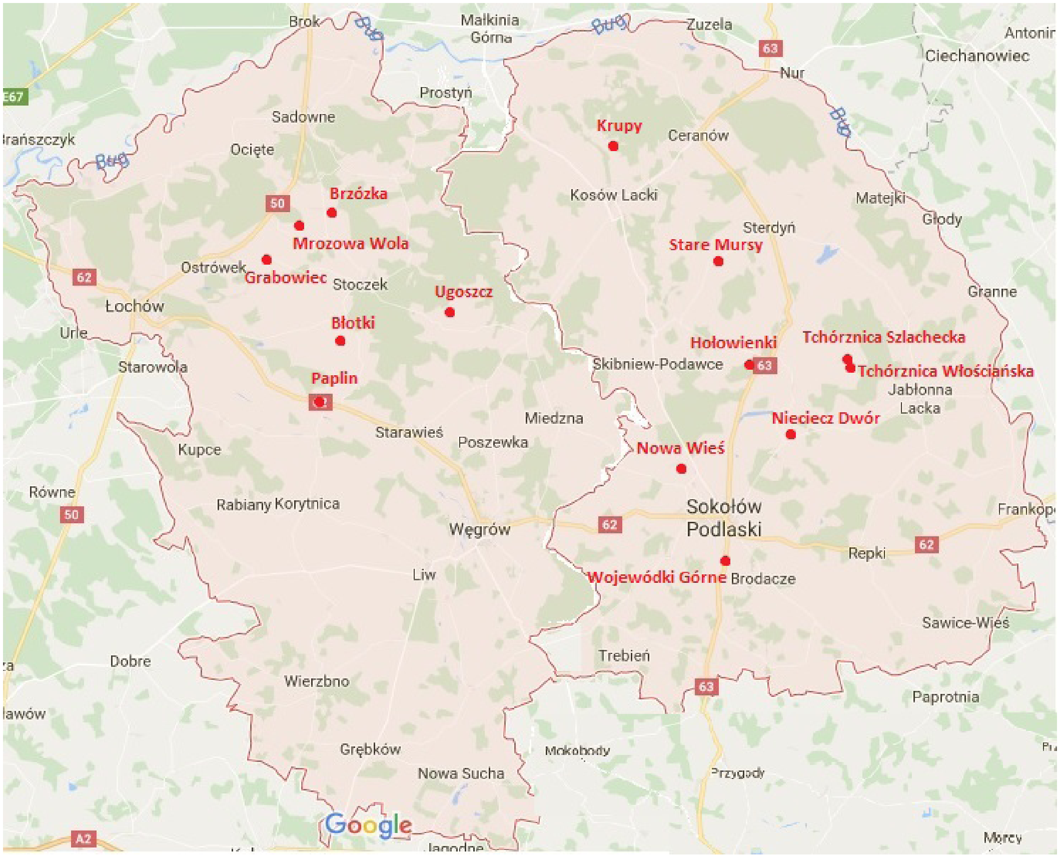

Figure 1.
Spatial distribution of the villages with the visited organic farms with livestock buildings in the Sokołów and Węgrów counties (by authors, with use of Google Maps)

12 buildings have only ground floor, two of them have also the second storey with full height, serving as storage part. Among the one-storey buildings, 11 have usable attic serving as a feed or bedding store.

Most of the buildings (36%) have the basement area between 100 and 150 m^2; 29% of buildings have the area under 100 m^2, 21% over 200 m^2, 14% between 150 and 200 m^2.

The analysis of construction of foundations showed that 64% of buildings have concrete continuous footing, 14% − ferroconcrete, 14% − stone-concrete, 8% − stone.

In the 5 buildings, the walls were erected with use of a full lime-sand brick. Slag hollow bricks were used to build 4 buildings. Full ceramic bricks and cellular concrete were used to erect walls in 4 buildings. 4 buildings have three-layered walls: in the 3 of them, the double full lime-sand brick wall with air gap was used, one building has the external layer made of the slag hollow bricks and the internal one – of the full lime-sand bricks. Some buildings were built of two and even three types of materials, hence the sum does not equal 14, i.e. the number of the investigated buildings (Figure 2).

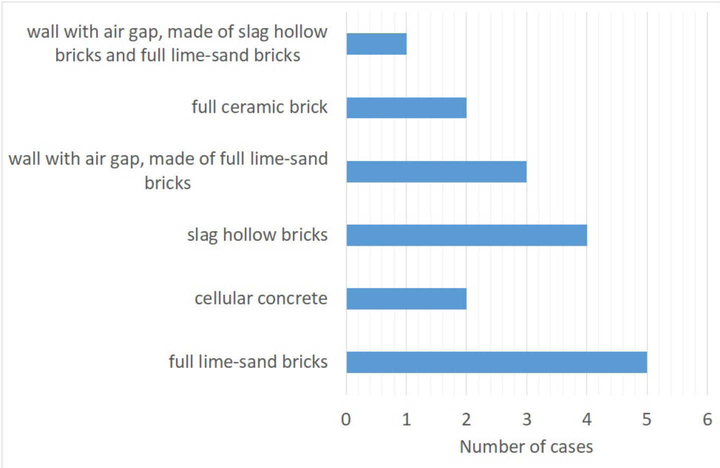

Figure 2.
Material used for construction of walls

Half of the analyzed buildings was covered by corrugated asbestos-cement boards, so-called eternit. The remaining buildings were covered with metal sheets: 29% (4 buildings) – the trapezoidal sheet, 21% (3 buildings) – the flat sheet (Figure 3).

For the gravitational ventilation, so-called ventilation chimneys in the roof ridge were used in the 8 buildings. In one of them, the chimney was made of wood, in the remaining 7 – of steel. Most of the buildings were equipped with at least 2 chimneys. Fresh air was supplied only through windows and gate. The rooms were lighted by glassed windows – 5–10 (in 50% of the buildings), over 10 (31%), under 5 in the remaining ones. Window frames mostly were made of metal (9 buildings); in the 4 buildings they were made of wood, in the one – of PVC.

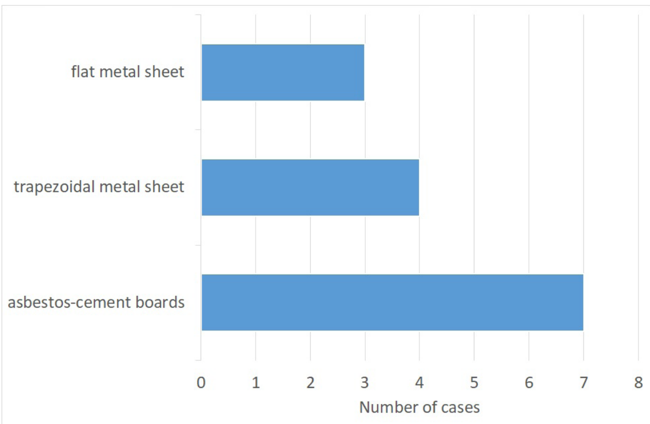

Figure 3.
Types of roofing

The majority (71%) of buildings were renovated or modernized. It consisted for example in exchange of roofing (4 buildings), window frames (4), expansion (3) or changes on pens for animals (3).

4. Discussion

In the source literature, quoted in the Introduction [14, 17–28], there are such materials used for construction of livestock building as silicates, porous ceramics, cellular concrete, unprocessed clay with straw, earth (pressed clay), wood, stone. These materials are considered as ecological (within the meaning of the objectives of the ecological building constructions referred to in the Introduction) but not free from defects – referring mainly to the process of their manufacturing or acquiring [36, 37].

During the investigations it was found that the materials that the livestock buildings in the investigated farms were made of were selected randomly, i.e. the building was erected with use of available raw materials without taking ecology into consideration. It results from the time difference – all buildings were constructed long before the switching into the organic way of farming. Difficulties, experienced in Poland till the end of 1990's, in accessibility to building materials caused that livestock buildings were erected with use of less processed materials (first of all, cheaper and more accessible, Figure 4). Paradoxically it is the reason that the livestock buildings in the

Figure 4.
Buildings subjected to analysis – selection

investigated farms are erected partly of ecological building materials, e.g. lime-sand brick, wood, full ceramic brick, cellular concrete.

According to the assumed criteria of the organic farming [38], the buildings in organic farms should be adopted at least in terms of ventilation, lighting and features of herd behavior of animals. The organic breeding satisfies basic needs of animals, mainly in terms of food, access to water, living space required, company of other animals, curing, maintenance hygiene, microclimate of rooms, light conditions. Simultaneously it prevents animals from mutilating themselves and ensures them a shelter against bad climate conditions. This term encompasses also conditions of transport of animals and humane slaughter [38]. The breeding in organic farms not only is aimed at increase of profits but also determines proper functioning of agri-ecosystem, i.e. a farm as a whole [39]. In the organic farm, a close connection exists between the animal breeding and soil – natural animal fertilizers, e.g. manure, excellently enrich the soil fertility and the lack of this connection evokes big environmental problems, such as lower crops. The animals in organic farms have to be healthy and productive, because their good health ensures good quality of animal products [40].

In the investigated farms, no substantial influence of the organic character of farming on the livestock buildings was found. It results from the fact that the conventional animal breeding is possible in certified organic farms.

An organic farm is or should be designed to function in a closed cycle when it comes to management of wastes generated in the production of food (of animal or plant origin).Ecological building construction, which objectives are a significant reduction of burden of buildings and their complexes on the natural environment, minimization of energy consumption during erection and exploitation of the buildings as well as the maximum use of the renewal energy [8], should be realized with use of ecological building materials. The investigations performed in another region of Poland and concerning dwelling houses in organic farms showed that the influence of the farm profile on constructive solutions and materials used is slight [33, 34].

However, the conclusions concerning the development of the organic farming in Poland [41] and stating that this farming is nowadays in the regression (systematic decrease of interest of this way of farming from years 2012−2013) are worrying. Reduction of the interest in the organic farming in Poland is quite unusual if compared to other European countries – minus 22% in the years 2012–2019 [4]. This issue should be thoroughly analyzed by economists or sociologists. From the point of view of the authors, this phenomenon is disadvantageous because the building substance will not improve but only remain in its unchanged form.

5. Conclusions

The organic farming along with the whole infrastructure is one of main directions of the European Green Deal, aimed at reconstruction of the economy of the EU toward the climate neutrality.

Based on the presented investigations, a conclusion can be drawn that the introduction and dissemination of the organic way of farming did not have any substantial influence on the livestock buildings in the Masovian voivodeship.

While switching into the organic way of farming, the farmers focused mainly at the production process – the transition period takes only plants and animals into consideration. Lack of direct requirements concerning construction of livestock or dwelling buildings becomes the reason of non-interference in the building's structure. However, there is a secondary correlation: the owners have nowadays the knowledge about the harmfulness or – at least – lack of environmental performance of some building materials (like, for example, asbestos-cement boards for roofing) and most of them declare fast changes in the future. It must be striven for application of ecological material and constructive solutions in all types of building investments.

The performed investigations confirmed the conclusions from the early 2000's that the building's structure in the organic farms does not change as fast as the agricultural production. The direct research realized in the organic farms is difficult due to high number of the registered entities which, in fact, do not carry out agricultural activity. There are relatively few farms leading the organic animal farming. Spatial sparseness of the farms also brings difficulties in carrying out the investigations. A farmers' distrust to researchers is also a serious barrier – the situation significantly deteriorated if compared to the research conducted 20 years ago, what seriously limits gathering of a wide comparative material.

Nowadays the organic farming in certain areas of Poland (and maybe of the world) can become the recommended way of the agricultural practice. These areas are, among others, buffer zones of national parks, landscape parks with their buffer zones, protected landscape areas, individual protection areas, e.g. ecological utilities.

A combination of an organic farm with ecological building construction has a high potential to bring measurable results connected to the Green Deal implementation.

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Conflicts of Interest

The authors declare no conflict of interest.

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Author Contributions

Conceptualization, N.D. and K.R., Methodology, N.D., Formal Analysis, N.D., Investigation, K.R., Resources, N.D., Data Curation, N.D., Writing – Original Draft Preparation, N.D., Writing – Review & Editing, K.R., Visualization, N.D and K.R., Supervision, N.D.

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Selected physico-chemical and microbiological parameters of water from the San River

Abstract: The aim of the study was to determine the microbiological and physico-chemical composition of water taken from the San River depending on the season. Water samples were taken in the winter and in the summer. The basic physico-chemical parameters of water samples were determined: pH using a pH electrode, conductivity and TDS (total dissolved solids) using a conductometric device, and turbidity using an infrared turbidimeter. In addition, water samples were diluted in the concentration range from 10^{-1} to 10^{-6} and inoculated by the surface plating method. The plates were incubated at temperatures 20°C and 37°C. After incubation, the number of microorganisms, such as bacteria and fungi was determined. Additionally, the Gram staining of microorganisms was performed.

The results obtained for pH, conductivity, TDS, and turbidity were within the expected parameters of natural surface waters. Microbiological analyses of samples taken in the winter and incubated at a temperature of 20°C showed the growth of G– cocci and bacilli bacteria and G+ bacilli bacteria, while at 37°C G– and G+ bacilli bacteria growth was observed. In the summer, no growth of microorganisms was noticed at a temperature of 20°C, but at 37°C G– cocci bacteria in chains were observed. However, growth of fungi was not observed.

Keywords: Microorganisms, Water, San River

1. Introduction

Water is an essential element of life on Earth. Most of the water resources are found in the oceans and seas. Among the water resources found on land, we can distinguish groundwater, rivers, and lakes. The most important factor shaping the physico-chemical composition of the

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flowing water is undoubtedly the geological structure of the rocks in which the river carries its channel. However, it is the meteorological conditions and human activity that determine the temporal variability of water quality [1–3]. Economic development and a significant increase in population cause degradation of the water ecosystem. The crucial anthropogenic factors influencing the quality of water ecosystems include municipal and industrial wastewater and, to a lesser extent, agriculture [4–6]. The greatest damage to the water environment can be seen in the sections of the catchment area located in cities where residential buildings, transport routes, and industry are dominant [7, 8]. Moreover, agriculture, using fertilizers in growing crops, contributes to changes in the chemical composition of ground and surface water [9].

Water is a diverse ecosystem in terms of species, which includes both unicellular organisms: bacteria, fungi, algae, etc., and multicellular organisms: insects, crayfish, fish. That large variety of species allows for water self-purification process which is used by man in the process of utilization of wastewater. That process is significantly influenced not only by the qualitative and quantitative compositions of microorganisms but also by physico-chemical parameters, such as content of organic and inorganic substances, pH, turbidity, salinity, which enable organisms to live. That is why it is so important to monitor both physico-chemical and biological parameters to ensure natural self-cleaning processes. Additionally, monitoring assists in safe use of water resources by people and animals. Early detection of the presence of pathogenic microorganisms prevents massive infections and occurrence of epidemic outbreaks [10].

The increase in water pollution makes it necessary to implement measures restoring the natural composition of water and preventing its further pollution. To achieve this aim, the European Parliament introduced the Directive 2000/60/EC [11], which requires that all member states take action to protect water. The directive contains guidelines specifying the scope and method of monitoring as well as the criteria for assessing the condition of water. Properly conducted monitoring of rivers provides knowledge about their ecological and chemical conditions, which are described on a five-point scale, where I means very good, and V means bad.

The aim of the study was to determine the microbiological and physico-chemical composition of water taken from the San River, dependent on the season. The research were conducted in the winter and summer of 2020. The analyses of compositions of surface water are the subject of numerous studies and publications around the world [12–22]. Since rivers are sources of drinking water, it is necessary to constantly monitor them and quickly react to the deterioration of flowing water quality.

2. Materials and methods

2.1. Study area

The San River is a tributary of the right bank of the Vistula. The river has its source near the village of Sianki in Ukraine. The total length of the river is 440 km, while the catchment area is 16,861 km^2, of which over 85% is within Poland. A significant part of the catchment area – due to the great natural and landscape values such as Bieszczadzki National Park, eight landscape parks, and three special bird protection areas Natura 2000 – is covered by legal protection.

The analysis of anthropogenic impacts in the rivers distinguishes 3 groups of surface waters: non-endangered (I class), endangered (II and III classes), and potentially endangered (IV and V classes) with the risk of failure to meet the Directive's objectives [23].

Regarding the San River catchment area, the percentage share of water in individual categories is as follows:

- non-endangered water – 80.3%
- potentially endangered water – 9.6%
- endangered water – 10.1%

From among the water catchment area of the San which are at risk of not meeting the objectives of the Water Framework Directive [11], 69% are waters with significant pressure from point sources of anthropogenic pollution (Figure 1 and 2) [23].

The greatest impact on the water quality of the San River catchment is made by municipal and industrial wastewater discharges, as well as by significant water demand for municipal and industrial purposes. Figure 1 shows the location of municipal and industrial water intakes, while Figure 2 shows the location of municipal and industrial sewage treatment plants.

Basing on the reports on the condition of the San River, it can be concluded that in the San catchment area there is a large load of pollutants of both municipal and industrial origin. Industrial wastewater is a large part of the emission of sewage from Sanok, Przemyśl, Leżajsk and Stalowa Wola [23].

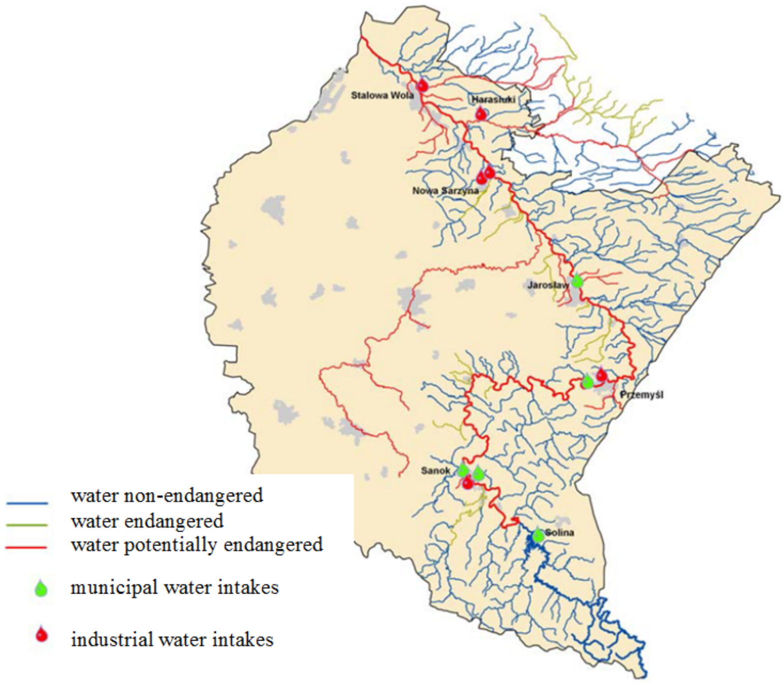

Figure 1.
Location of municipal and industrial water intakes

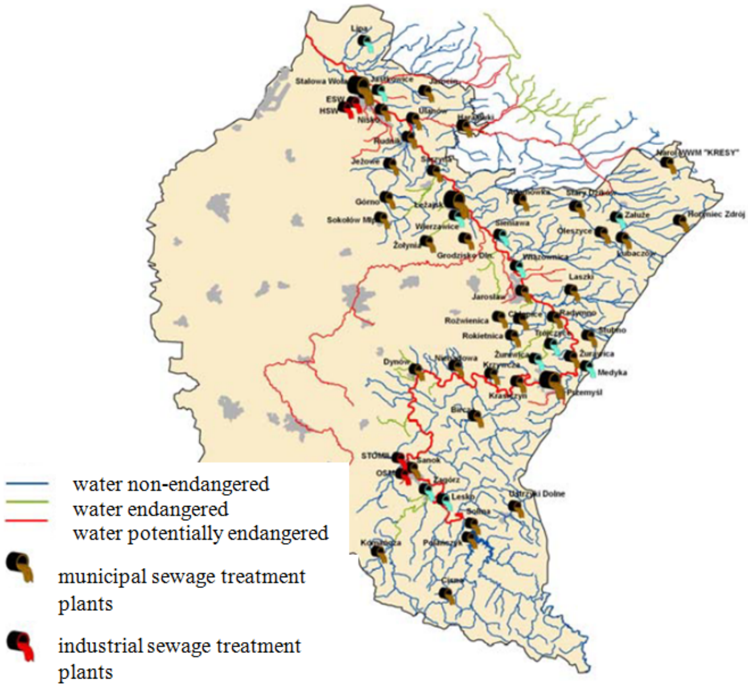

Figure 2.
Location of municipal and industrial sewage treatment plants

2.2. Sampling design

Water quality tests were carried out in February and June of 2020 in the city of Stalowa Wola, where there are two wastewater treatment plants, from which sewage is discharged through one common channel into the San River.

The location of the sampling sites is shown in Figure 3. Water samples were taken upstream from the water treatment plant channel.

Single samples of water were taken directly from the river at a depth of 50 cm into clean glass stoppered sampling bottles. Analysis of water quality was done in sterile bottles, carefully cleaned, and rinsed thoroughly with distilled water. The physico-chemical composition was tested in the laboratory with proper equipment: pH level was determined with Elmetron CPC-505 pH meter, Electric Conductivity (EC) and Total Dissolved Solids (TDS) were measured with Hama Instruments HI 9813-5 device, and sample turbidity was tested with CyberScan TN100 infrared turbidimeter.

Microbiological analyses were preceded by serial dilutions of water samples which were prepared from 10^0 to 10^{-9} in 10 ml of sterile 0.9% of sodium chloride and inoculated on agar. Plates were incubated at 20°C and 37°C for 48 hours. Colonies were counted after 48 hours of incubation. Then, bacteria cells were stained with Gram's method.

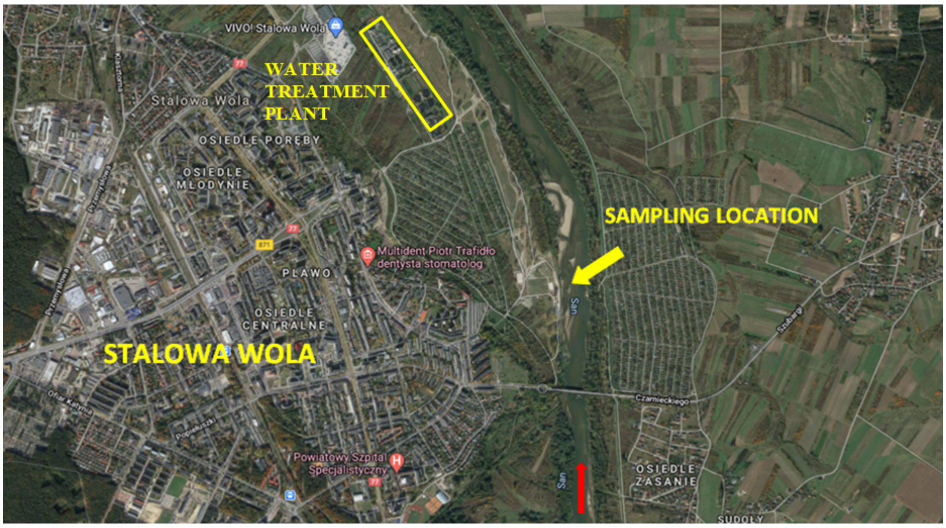

Figure 3.
Location of the sampling site

3. Results

The collected data (Table 1) show a decrease in pH, EC, and TDS levels in the June sample compared to the February sample, while turbidity level increased almost 5 times in the June sample.

Table 1.
The results of basic physico-chemical tests

Collection date	pH	EC [mS/cm]	TDS [mg/dm^3]	Turbidity [NTU]
February 24th	8.30	0.55	363	6.6
June 22nd	8.00	0.38	263	25.9

In the present study, in the winter samples, the growth of microbial colonies was noted after incubation at temperatures of 20°C and 37°C. The highest number of bacteria was observed on undiluted samples. Next, the number of bacteria decreased with an increasing dilution of water samples. In two dilutions, 10^{-1} and 10^{-2}, the number of colonies was reduced. At dilutions from 10^{-3} to 10^{-6}, no colony growth was observed at temperatures of 20°C and 37°C. Microorganisms incubated at both temperatures were collected from the plates and stained with Gram's method.

Among the microorganisms incubated at 20°C, Gram-negative cocci, as well as Gram-positive bacilli and Gram-negative bacilli (Figure 4) were observed. On the other hand, in the plates incubated at 37°C, Gram positive and Gram-negative bacilli were observed (Figure 5).

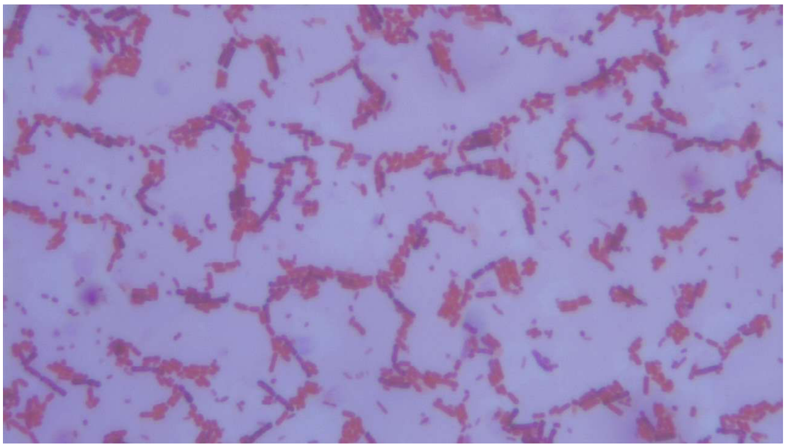

Figure 4.
Microorganisms incubated at temperature of 20°C, magnification 1000×

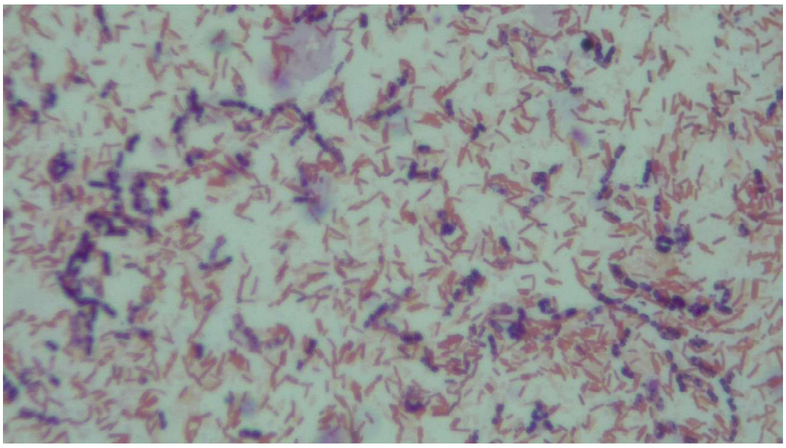

Figure 5.
Microorganisms incubated at temperature of 37°C, magnification 1000×

In the summer samples, incubated at 20°C, no growth of colonies of microorganisms was noted. A growth of microbial colonies occurred after incubation at a temperature of 37°C. The highest number of bacteria on undiluted samples was observed. Then, the number of bacteria decreased with increasing dilution. In two dilutions, 10^{-1} and 10^{-2}, the number of colonies was

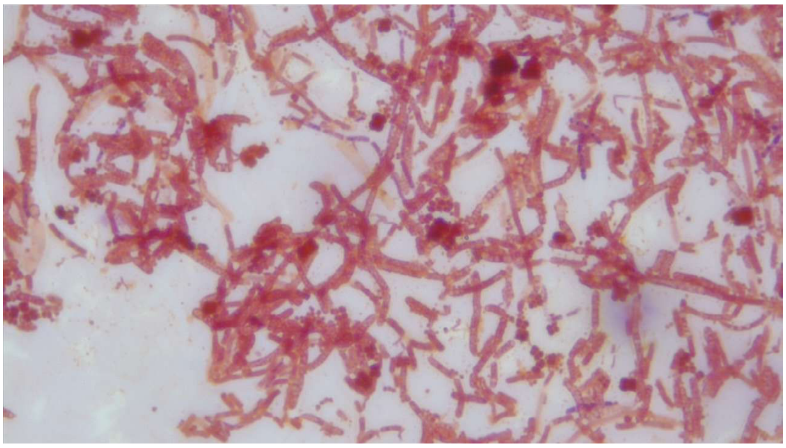

Figure 6.
Microorganisms incubated at temperature of 20°C, magnification 1000×

reduced. The microorganisms were collected from the plate incubated at 37°C and stained with Gram's method. In microscopic images, an increase in Gram-negative cocci in the chains was observed (Figure 6).

4. Discussion

Water is an environment for many organisms. Some of them are microflora for which water is the primary environment. The others belong to the allochthonous microflora.

The composition of the indigenous microflora depends on the availability of nutrients, oxygen content, temperature, and pH. Their content is typical for aquatic ecosystem. This group of microorganisms includes: psychrotrophs and psychrophiles. These microorganisms live in low temperatures and play important role for the ecosystem. They play an important role in the mineralization of organic substances, which leads to the self-purification of rivers. Additionally, they play an important role in the food chain.

Allochthonous microflora – incorporated, occurs periodically in water. Its source may be treated sewage discharged into water or soil.

For selected markers, in the winter and summer which means that the EC, TDS and pH levels do not exceed the ranges established in the regulation (Table 2) [25] ensuring the functioning of a specific type of ecosystem.

The decrease in pH, EC, and TDS levels, between winter and summer samples, could be linked to increased rainfall causing an influx of clean water and reducing the concentration of any dissolved substances in the river. The same rainfall could increase turbidity because surface runoff from adjacent land with suspended undissolved materials had been flowing directly into the river

Table 2.
Comparison of sample results with border values of physico-chemical quality markers assessment of surface waters according to Polish regulation [25]

	pH	EC [mS/cm]	TDS [mg/dm^3]	Turbidity [NTU]
February 24th	8.30	0.55	363	6.6
June 22nd	8.00	0.38	263	25.9
Class I water	7.70–8.40	≤0.753	≤ 474	n/a*
Class II water	7.50–8.40	≤0.850	≤ 525	n/a*
Class III-V water	n/a**	n/a**	n/a**	n/a*

* Turbidity is not a marker for water quality assessment in Polish regulation.
** Border values are not fixed for classes higher than II for these markers in Polish regulation.

or its tributaries. During rainy season, particles suspended in water tend to be in motion due to a higher rate of circulation, whereas in dry season particles tend to settle a little faster. It is also possible that the increased phytoplankton growth in the summer compared to the winter could be another reason for decreased EC and TDS levels as microorganisms absorb dissolved nutrients and convert them into biomass, which in turn increases water turbidity.

As was shown in our study, in the winter, an increase in both psychrotrophs and mesophils bacteria was observed. Fungi growth was also noted. Among the psychrotrophs, Gram-negative cocci as well as Gram-positive and Gram-negative bacilli bacteria were observed. In contrast, Gram-positive and Gram-negative bacilli bacteria were observed among the mesophils. These bacteria (Gram-negative bacilli bacteria) belong to the group of microorganisms colonizing the digestive tract of humans and animals. Their increase in the winter may indicate contamination of the water with feces. On the other hand, in the summer, among the mesophilic bacteria, an increase in Gram-negative bacilli bacteria in chains was observed, but there was no increase in the psychrotrophs.

The results obtained allow us to conclude that temperature is a strong factor which limits the development of microflora. The occurrence of psychrophilic bacteria in the San River may be associated with the presence of organic substances, which is caused by the discharge of sewage sludge into the water. On the other hand, the existence of mesophilic bacteria, especially from the coli group, may indicate fecal contamination. Their presence may also result from disturbing surface water sediments. An important factor, not researched in this study but noted in archival research, is the presence of toxic metals Sn, Cd, Cu, Ni, Pb, Hg, Zn coming from wastewater. Along with the factors mentioned above, i.e., temperature and the presence of nutrients, the toxic metals significantly affect the composition of the microflora of rivers.

All biotic and abiotic factors relate to the formation of microflora in the river. However, contamination of water significantly disturbs the ecological balance and deteriorates the condition of water. Therefore, monitoring of both physico-chemical and microbiological parameters is necessary as it helps to prevent degradation of the natural environment.

5. Conclusions

Based on the research, it is possible to observe that the results obtained are clearly different during the two seasons. Undoubtedly, this is the result of significantly different climatic features. Microbiological tests in the winter revealed an increase in psychrotrophs, mesophils and fungi. Also, Gram-negative bacteria have been reported in the digestive tract in humans and animals which have been associated with water contamination. In the summer, however, no increase in psychrotrophs was observed. In February, the pH becomes more alkaline, which may be due to the direct inflow of water from precipitation and from the melting snow. The electrical conductivity usually increases during snowfall and frost, while it decreases in summer during prolonged and intense rainfall. In the winter, salinity in water is higher than in the summer. On the other hand, the measured water turbidity showed a considerable discrepancy in the results. The changes are related to the flushing of soil particles and to their intensity of movement in the riverbed. In the winter, the soil covered with rotting organic debris does not offer much resistance to meltwater or rainwater. In the summer, heavy rainfall increases the flushing efficiency.

Continuous monitoring of the San River water quality is necessary to develop a water quality improvement plan for that river.

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*Iwona DESKA**

The impact of the mosses growth on the water retention capacity of green roofs

Abstract: The urbanization and the population growth lead to an increase of the share of impermeable surfaces in the cities and, as a consequence, to an increase of flood risk. Therefore, there is a need for application of the solutions that can support the operation of traditional drainage systems in the urban areas. Examples of such solutions can be green roofs. The goal of the research was to investigate the retention capacity of seven extensive green roof modules. In the case of five modules the substrates were amended with hydrogel (the cross-linked potassium polyacrylate). Six modules were vegetated with goldmoss stonecrop (*Sedum Acre*). Additionally, all vegetated modules were partially covered with mosses. The share of areas covered with mosses ranged from 6.72% (in the case of relatively new module) to 43.04% (in the case of the oldest vegetated module containing hydrogel). The obtained results show that the green roofs may have a positive effect on the stormwater management. The best retention capacity was exhibited by the relatively new modules containing the plants and hydrogel admixtures. On the other hand, the lowest retention capacity was exhibited by the oldest module mostly covered by mosses. The obtained results suggest that the overgrowth of mosses on the green roof may decrease their retention capacity under certain atmospheric conditions (especially at low temperatures and high levels of the air relative humidity).

Keywords: green roofs, water retention capacity, stormwater management, moss

1. Introduction

The process of urbanisation leads to the persistent sealing of the drainage basin surface in the cities [1] and to the transformation of the hydrological processes occurring in the urban catchments [2]. A large part of natural areas are displaced by impervious areas, especially by buildings, streets, pavements, places [3, 4]. We are also dealing with an increase of the frequency, intensity and duration of heavy rainfalls due to the climate changes [5]. Both the climate changes

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and urbanisation may lead to an increase of flood risk [1]. More and more often, in the cities there is a need for application of the Sustainable Drainage Systems (SuDS) (or Sustainable Urban Drainage Systems (SUDS)) that can support the operation of the traditional sewerage systems [1, 4, 6, 7]. SuDS comprise techniques and technologies used to drain stormwater in a more sustainable manner than traditional sewerage systems [7]. The primary purpose of using SuDS is to diminish the share of impervious surfaces in urban areas and to replicate as well as possible the natural rainwater drainage and infiltration [6, 7]. The SuDS devices are classified as components of the green infrastructure (GI). The examples of SuDS are, among others, bioretention systems (such as rain gardens), and green roofs [8].

Green roofs can be grouped into the source control devices. Their function is to slow down, as well as store and treat the runoff at locations close to where precipitation has fallen [9]. Other source control measures are: permeable pavements , subsurface storage measures, rain gardens, rainwater harvesting measures as well as disconnected downpipes [6, 9].

Green roofs are the layered constructions covered with substrate (the artificial, specially prepared soil) and plants [8]. Taking into account the construction, substrate properties and thickness, the roof weight, the vegetation type, the way of use as well as operation and maintenance, green roofs can be divided into intensive, simple intensive, as well as extensive roofs [8, 10]. Because of costs and the building weight restrictions, the extensive green roofs are much more common than the intensive and semi-intensive roofs [11]. Extensive green roofs are much more common due to the lower construction costs, relatively low weight as well as shallow substrates and plants that are drought tolerant [8, 10].

Extensive green roofs provide many ecological and economic benefits [8, 11–13]. One of them is the energy conservation [14, 15]. Green roofs are a potential energy-saving strategy and thus they have been adopted in some countries [16, 17]. Vegetated roofs have the potential to improve the building's energy behaviour and leads to a decrease of both the cooling energy demand in the summer and heating energy demand in the winter [18]. Very important benefit of green roofs is the reduction of the high temperatures in cities during the summer and thus mitigating the "urban heat island" effect [18–20]. The green roofs can contribute to the atmospheric CO_2 reduction [19, 21]. However, one of the most important functions of green roofs is the stormwater management [22]. Green roofs contribute to reduction the intensity of the stormwater runoff due to the evapotranspiration, thereby reducing the risk of flooding in urban areas [3, 22–24].

The important aspects of green roofs, which very strongly influence their hydrological performance, (and thus the retention capacity) are: the roof type (e.g. intensive, extensive), their construction and type of used materials (e.g. drainage elements), the substrate type and thickness, the roof slope, the type, age and condition of vegetation. The additional parameters which have impact on the green roof retention are: the season, weather (e.g. air temperature and relative humidity), antecedent dry weather period (ADWP), the initial substrate conditions at the beginning of precipitation (e.g. the substrate moisture), and the rainfall characteristics (e.g. the rainfall depth, intensity and duration) [3, 4].

Very important component of green roof is the substrate. It should be relatively light and good permeable [25]. The substrate provides the physical support and supply water and nutrients to plants [26, 27]. Extensive green roofs usually have the thin substrate layers and thus they can be exposed to drought, especially in the summer. The substrate can be amended with different additives e.g. superabsorbent polymers (SAPs) that may improve the conditions for vegetation, especially increase the availability of water to plants [28, 29]. The example of SAP (which can be also called hydrogel) can be cross-linked potassium polyacrylate [28, 30]. Hydrogel may absorb considerable amount of water (even to several hundred times more than its dry mass) [30]. This water can be available for plants during the drought.

The plants which are commonly used on the extensive green roofs are sedum which can survive in the harsh environment. Grasses and herbs are also used very often as the vegetation layer [24, 31]. However, it often happens that the mosses spontaneously colonize and overgrow the green roofs [31, 32]. It should be noted that the intentional planting of mosses on green roofs is less common [31]. The mosses may contribute to the biodiversity on green roofs. Cruz de Carvalho et al. in their research conducted in 2019 [33] have proposed the method of selection the moss species that can grow on green roofs under different local weather conditions. Heim et al. (2014) in their work suggest that the mosses may both facilitate and inhibit the growth of selected vascular plants. They suggest that mosses have a potential to reduce the soil temperature and thus can influence the evapotranspiration from green roof [31]. Paço et al. (2019) in their research have proposed that good solution can be planting of green roof with a mixture of vascular plants and mosses, where mosses can exhibit a larger water retention and the vascular plants can use the water accumulated by mosses [34].

The goal of the research described in this paper was the investigation of the water retention capacity of 7 green roof modules of which 6 were spontaneously colonized by mosses. Additionally, in the case of 5 modules the substrates were partially amended with hydrogel (the cross-linked potassium polyacrylate).

2. Materials and methods

The research was conducted from 25.10.2019 to 19.01.2020 under natural weather conditions. The experiments have been carried out within the Faculty of Infrastructure and Environment (Czestochowa University of Technology). The green roof modules were constructed with use of garden trays that were placed with a slope equal to 5%. In the right lower corner of each garden tray was drilled the hole to which was attached a flexible PVC hose in order that the excess of rainwater would outflow from the module. Each experimental platform consisted of following components: the drainage element Floradrain FD 25 (55.7 × 55.7 cm), the filter sheet SF (70 × 70 cm), the properly prepared extensive substrate "Sedum carpet" produced by ZinCo (with or without hydrogel admixture), the plants (optionally). The detailed cross-sections of the green roof modules are described in the Figure 1.

The modules 1 and 2 were constructed in 2017 (March), modules 3 and 4 – in 2017 (November), modules 5 and 6 in 2018 (April), and module 7 in 2019 (October). The substrates on modules 1, 2, 3, 4, and 7 were built of 2 layers. The upper layer consisted of 5 kg of substrate "Sedum carpet". The lower layer consisted of 6 kg the same substrate amended with 0.5% by weight of hydrogel (the cross-linked potassium polyacrylate). The growing media on modules 5 and 6 were

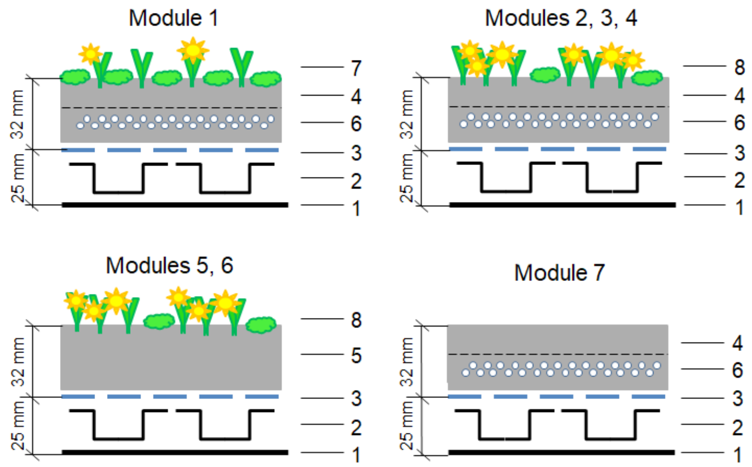

Figure 1.
The cross-sections of the green roof modules
1) the garden tray bottom, 2) the drainage element Floradrain FD 25, 3) the filter sheet SF,
4) 5 kg of the extensive substrate „Sedum carpet", 5) 11 kg of the extensive substrate „Sedum carpet",
6) 6 kg of the extensive substrate „Sedum carpet" with admixture of 0.5% by weight of hydrogel,
7) the plants (mostly the mosses and partially the goldmoss stonecrop Sedum Acre),
8) the plants (mostly goldmoss stonecrop)

uniform and consisted of 11 kg of substrate "Sedum carpet" without amendments. The substrate "Sedum carpet" consisted of the high-quality crushed brick, mineral aggregates (i.a. expanded clay), wood chips, sandy soil enriched with compost and fibre materials, etc. The average substrate surface area in each green roof module was equal to 3213.3±17.2 cm^2. The average total thickness of each substrate was relatively small and amounted to about 3.2±0.1 cm. It should be emphasized that so small substrate thickness can be found e.g. in case of green roofs created with use of pre-grown vegetation mats. Modules 1–6 were vegetated while module 7 was not vegetated. The vegetation which was originally used at the stage of the module construction was goldmoss stonecrop (*Sedum Acre*). The composition of the vegetation changed during the operation of the modules. The substrates on modules 1–6 (especially module 1) were partially supplanted by mosses (especially in 2019). The percentage of mosses in the entire area of each module (in November–December 2019) shows Table 1.

Table 1.
The percentage of mosses on the green roof modules

The module number	1	2	3	4	5	6	7
Percentage of mosses [%]	43.04	10.87	8.54	6.72	7.32	8.08	0
Area of mosses [cm^2]	1 382.90	349.30	274.50	215.80	235.20	259.50	0

The investigations of the water retention rate of green roof modules were carried out involving simulated and natural precipitations. The volume of water retained on each module was established based on the difference between the volume of rain falling on the module surface and volume of runoff (Equation 1):

$$S = P - R \ [\mathrm{cm}^3] \tag{1}$$

where:

- S – volume of water retained on green roof module [cm^3],
- P – volume of precipitation that is falling on the module surface [cm^3],
- R – volume of the runoff [cm^3].

The runoff coefficient was calculated on the base of ratio of runoff volume R to the volume of precipitation P falling on the module surface (Equation 2):

$$\psi = \frac{R}{P} \cdot 100\% \tag{2}$$

where:

- ψ – runoff coefficient [%],
- P – volume of precipitation that is falling on the module surface [cm^3],
- R – volume of the runoff [cm^3].

The parameters of atmospheric conditions prevailing during investigations and the rain depths were recorded with use of the weather station Vantage Pro2 (Davis Instruments, Hayward, USA) at 10-minute time intervals.

3. Results

The parameters of natural and simulated precipitations (the antecedent dry weather period (ADWP), rain depth, rain volume P, mean air temperature prevailing during ADWP, mean air humidity prevailing during ADWP) are given in the Table 2.

The depths of the natural rainfalls, recorded during the whole period of investigations (from 20.10.2019 to 22.01.2020) with use of the weather station are presented in the Figure 2. The graph also shows the calculated depths of simulated precipitations. Not all of these precipitations were included in the analysis.

The Table 3 presents the volumes of rainwater which were retained within the structures of green roof modules during experiments (S), calculated on the base or runoff using Equation 1. The Table 4 presents the values of runoff coefficients (ψ) calculated based on Equation 2.

Table 2.
The properties of natural and simulated precipitations

Dates of rain events	ADWP	Rain depth	Rain volume	Mean air tempera-ture	Mean air humidity	Rain type
	d:h:min	mm	cm^3	°C	%	–
25.10.2019	1:13:00	7.8±0.1	2 500±15	14.6	69.3	simulated
12.11.2019–13.11.2019	1:19:10	4.6±0.2	1 478±77	5.8	87.4	natural
13.11.2019	0:05:10	3.0±0.2	964±77	8.0	95.1	natural
16.11.2019–19.11.2019	2:05:40	0.8±0.2	257±77	6.0	94.6	natural
19.11.2019	0:05:10	10.9±0.1	3 500±20	7.9	79.6	simulated
21.11.2019	2:02:20	2.4±0.2	771±77	8.1	88.0	natural
08.12.2019–10.12.2019	3:05:00	1.4±0.2	450±77	–0.6	79.9	natural
14.12.2019–15.12.2019	1:02:00	1.6±0.2	514±77	3.7	77.0	natural
08.01.2020–11.01.2020	3:00:20	3.0±0.2	964±77	0.2	86.6	natural
18.01.2020–19.01.2020	1:06:50	1.4±0.2	450±77	2.3	89.2	natural

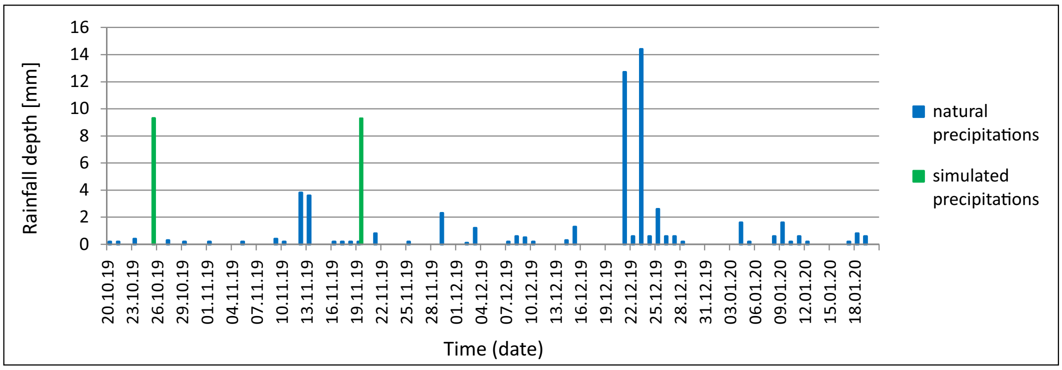

Figure 2.
The depths of the natural and simulated precipitations

Values given in the Table 4 vary considerably, from 0.0% to 97.8%. The reason of such situation is that the investigations were conducted mostly with use of natural precipitations. The depths, intensities, and durations of them as well as antecedent dry weather periods (and thus retention depths) were diversified. It should be noted that the meaningful comparison of runoff coefficients is possible only when the data established during the same precipitation are taken into account.

Table 3.
The volumes of rainwater retained within the green roof modules

Dates of rain events	Volume of water retained within the green roof modules S [cm^3]						
	1	2	3	4	5	6	7
25.10.2019	1 326±22	1 599±21	1 362±22	1 399±22	1 384±22	1 480±21	957±25
12.11.2019–13.11.2019	1 354±79	1 422±78	1 360±79	1 362±79	1 316±79	1 354±79	1 264±79
13.11.2019	45±83	185±83	202±83	197±83	134±83	134±83	140±83
16.11.2019–19.11.2019	251±78	251±78	251±78	251±78	255±78	253±78	255±78
19.11.2019	1 758±31	1 782±31	1 748±31	1 712±31	1 572±31	1 600±30	1 768±31
21.11.2019	17±83	255±83	241±83	229±83	217±83	225±83	489±80
08.12.2019–10.12.2019	300±78	380±78	381±78	380±78	386±77	386±77	48±80
14.12.2019–15.12.2019	248±80	469±78	512±78	514±77	494±78	514±77	254±80
08.01.2020–11.01.2020	588±81	730±79	782±79	752±79	680±80	764±79	706±80
18.01.2020–19.01.2020	405±78	444±78	446±78	444±78	448±78	449±78	440±78

Table 4.
The values of the runoff coefficients ψ

Dates of rain events	The runoff coefficients for the green roof modules ψ [%]						
	1	2	3	4	5	6	7
25.10.2019	47.0±0.6	36.0±0.5	45.0±0.6	44.0±0.5	44.6±0.5	40.8±0.5	61.7±0.8
12.11.2019–13.11.2019	8.4±0.6	3.8±0.3	8.0±0.6	7.8±0.5	11.0±0.7	8.4±0.6	14.5±0.9
13.11.2019	95.3±8.2	80.8±7.1	79.0±6.9	79.6±7.0	86.1±7.5	86.1±7.5	85.5±7.4
16.11.2019–19.11.2019	2.3±1.1	2.3±1.1	2.3±1.1	2.3±1.1	0.8±0.6	1.6±0.9	0.8±0.6
19.11.2019	49.8±0.6	49.1±0.6	50.1±0.6	51.1±0.6	55.1±0.6	54.3±0.6	49.5±0.6
21.11.2019	97.8±10.5	66.9±7.5	68.7±7.6	70.3±7.8	71.8±8.0	70.8±7.8	36.6±4.0
08.12.2019–10.12.2019	22.3±4.7	1.6±0.6	1.3±0.5	1.6±0.6	0.0±0.0	0.0±0.0	87.7±18.3
14.12.2019–15.12.2019	51.7±8.3	8.8±1.5	0.4±0.3	0.0±0.0	3.9±0.8	0.0±0.0	50.6±8.2
08.01.2020–11.01.2020	39.0±3.5	24.3±2.1	18.9±1.7	22.0±2.0	29.5±2.7	20.7±1.9	26.8±2.4
18.01.2020–19.01.2020	10.0±1.9	1.3±0.5	0.9±0.4	1.3±0.5	0.4±0.5	0.2±0.3	2.2±0.6

The comparison of runoff coefficients obtained during different rainfalls has disadvantages, because their values depend not only on green roof retention capacity but, to a large extent, on both the rain depth and green roof modules conditions at the beginning of precipitation, resulting from ADWP duration (e.g. the substrate moisture and the filling degree of drainage mat buckets). Therefore it is possible that the higher value of runoff coefficient can be obtained in case of lower precipitation depth (and vice versa). Therefore, the indirect interpretation of results showed in the Table 4 can be very difficult. The basic role of Table 4 is to compare the runoff coefficients obtained during the same precipitations and to show the wide variety of their values during different rainfalls. The analysis of all obtained results of runoff coefficients for 7 modules is presented in the Figure 3 which demonstrates the box-whisker plots showing their minimum, average, and maximum values, as well as values of 1st, 2nd and 3rd quartiles. The aim of the analysis presented in Figure 3 is to select the modules having the highest runoff coefficients on grounds of results obtained during all precipitations described in the Table 2.

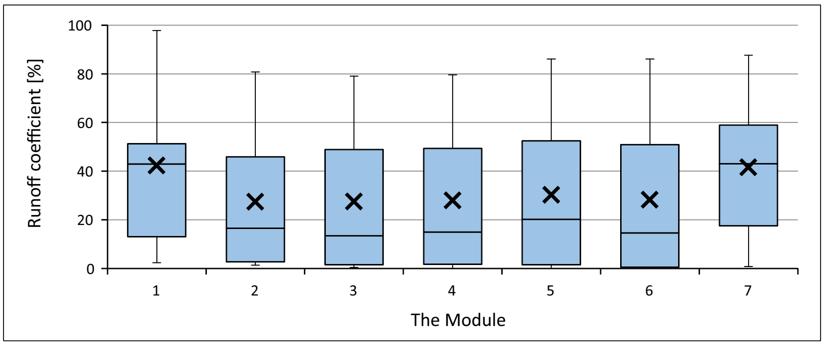

Figure 3.
The non-parametric distribution of the runoff coefficients (ψ); the boxes represent 1st, 2nd and 3rd quartiles; n = 10

The results presented in the Figure 3 show that the highest maximal value of runoff coefficient was obtained in the case of module 1 (in the case of which 43.04% of surface was supplanted by mosses). However, it should be noted that relatively high runoff coefficients were obtained in the case of module 7 (which not contained the plant layer). The average values of runoff coefficient in the case of modules 1 and 7 were similar and, simultaneously, close to the median values of this parameter. In the case of remaining modules (2–6) the average and median values of ψ coefficient were lower than these in the case of modules 1 and 7. Obtained results suggest that the expansion of mosses on the green roof may diminish its retention capacity, especially in cold months, at low air temperatures and high values of relative humidity. The results of water retention capacity (expressed in terms of runoff coefficient) obtained in the case of module heavily supplanted by mosses were similar to the results obtained in the case of module without plants.

4. Discussion

The obtained results suggest that the mosses expansion on the green roofs can diminish their water retention capacity in the autumn and winter, especially at relatively air temperatures and the high values of relative humidity. On the base of the results given in the Tables 3 and 4 it can be concluded that in the majority cases the lowest values of water retention capacity and thus the highest values of runoff coefficient were obtained in the case of modules 1 (with vegetation layer supplanted by mosses) and 7 (not containing the plants).

The obtained results and the observations of modules conducted during experiments allow to conclude that the mosses can influence the growth of the neighboring species (in this case the goldmoss stonecrop). This conclusion agree with those formulated by Heim et al. (2014) [31] who in their research analyzed the influence of mosses on the neighboring vascular plats. They concluded that the mosses may have both positive or negative influence on the vascular plants. In the case of their research as well as in the case of investigations described in the current article the influence of mosses on the goldmoss stonecrop (*Sedum Acre*) was negative. The similar results were obtained by Kleinhesselink and Cushman who in their research conducted in 2019 [35] found that the effects of bryophyte on vascular plants can range from negative to positive. Lönnqvist et al. (2021) in their research [36] found that the richness of species on the green roof can be significantly and negatively correlated with moss cover. The reason for this is that the mosses can survive in harsh and drought environments because they can bind the water from atmosphere but, simultaneously, they inhibit the germination of vascular plants and prevent their recolonization after the long drought periods. It is possible that it was probably the cause of the expansion of mosses and loss of relatively great part of goldmoss stonecrop on the module 1. Additionally, Lönnqvist et al. reported that the substrate depth can be positively correlated with vascular plant cover. It means that the low thickness of the substrate (like in the case of current research) can be the reason of moss expansion. Burszta-Adamiak et al. in the research conducted in 2019 [32] have confirmed that the mosses have the ability to colonize the green roofs and may survive in harsh drought conditions. They concluded that mosses can form the long-lasting green cover and can be an optimal solution for the improvement of urban hydrology [32].

Taking into account the results of investigations described in current paper, it should be stressed that the retention of module 1, heavily supplanted by mosses, was relatively low. On the other hand, it is well known that the mosses have high ability to retain water. It seems that this feature may have a negative impact on the retention capacity of vegetated roofs in autumn and winter, when the relative humidity of air is relatively high and the air temperature is relatively low. It can be supposed that at high values of relative humidity the mosses have condensed the water from the atmosphere vapor and this situation probably led to higher substrate moisture and to partially filling of drainage mat buckets. And, as a result, the retention capacity of module 1 was lower than that of modules 2–6. The same rule should not take place in late spring and summer when the air humidity is low and the mosses bind less water from the atmosphere.

These results are consistent with the conclusions formulated by Lönnqvist et al. (2021). They reported that the mosses can cool the substrate as well as bind the water which influences the evapotranspiration and results in higher substrate moisture content [36]. It is in line with the findings of Heim et al. who reported that the mosses may increase the substrate moisture [31]. It should be noted that the research described in the current paper was conducted in the autumn and in the winter. There is a need to continue the experiment during the spring and summer, at the higher air temperatures and lower values of relative humidity.

It should be emphasized that thicknesses of substrates used in experiments were relatively small. Such small thicknesses are found, inter alia, in case of green roofs created with use of pre-grown vegetation mats. Despite the fact that typical extensive green roofs built using the traditional method usually have greater thicknesses, one of additional research goals was to examine the hydrologic performance of low-thickness growing medium. The advantage of small substrate thickness can be, inter alia, the low weight of the roof. But, on the other hand, such solution has disadvantages, may pose problems, and has a negative impact on green roof hydrological performance, especially resulting in relatively small retention capacity. The next problem concerning the low-thickness substrate can be worse thermal performance of the green roof due to the lower thermal insulation and heat loss in winter as well as faster water loss resulting in decrease of cooling effect in summer [17]. It should be noted that the water evaporation (and evapotranspiration) is one of most important mechanisms of lowering the temperature of green roof [17]. Based on conducted research results it can be ascertain that in case of such a small thickness the vascular plants vegetation conditions may be unfavourable, especially in times of drought. This can be reason for such an intensive development of mosses and partial disappearance of sedum (Sedum Acre), especially on module 1 which contained the oldest vascular plants.

5. Conclusions

The results of the conducted research allow to formulate the following conclusions:

1. The green roofs, as a part of Green Infrastructure can be an effective tool for the stormwater management in the urban areas. The obtained values of runoff coefficients were different but in many cases they were relatively low despite the fact that the substrates on the modules had the low thickness.
2. The best water retention capacity (the lowest runoff coefficient) was exhibited by the modules with the relatively small share of mosses (2, 3, and 4), containing both the plants and hydrogel. Relatively good results were obtained in the case of modules containing plants, with substrates not amended with hydrogel (5 and 6).
3. The lowest retention capacity (and thus the greatest runoff coefficient) was exhibited by the oldest module 1, mostly covered by mosses and the module 7, without any plants. The results suggest that the overgrowth of mosses on green roofs may decrease their water retention capacity in the autumn and winter at relatively low temperatures and rather high values of relative humidity due to the fact that mosses can bind the water from atmosphere and have an ability to increase the substrate moisture and filling of drainage mat buckets during the antecedent dry weather period.

4. On the base of the research it can be concluded that the mosses can spontaneously overgrow the green roofs and may have an unfavorable impact on the germination and growth of vascular plants. The additional cause of the mosses expansion can be the low thickness of substrate which is unfavorable for the vascular plants, especially in times of drought.
5. There is a need to conduct the further experiments under different weather conditions (e.g. in the spring and in the summer, at the higher air temperatures and lower values of relative humidity) to assess the hydrologic performance of green roofs overgrown by mosses at such conditions.

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Green synthesis of nanomaterials for Eye Cancer research with focus on Retinoblastoma

Abstract: Targeted cancer therapy using eco-friendly nanomaterials has gained importance recently. Nano-conjugates containing peptide, polymer, toxin and aptamer are developed as therapeutic moieties for targeted cancer therapy sparing normal cells. This would reduce off target effects associated with cancer therapy. Therapeutic drug delivery for various ocular diseases is challenging due to unique structure, physiology, and function of the eye. Several nanotechnological approaches are available that could address this area. Currently there are no approved nanodrugs for ocular cancers. Retinoblastoma (RB) is paediatric cancer that is prevalent 1 in 15 000–20 000 births and is more fatal in developing countries and secondary tumors in number of organs such as lung bladder and brain are reported later in the life. There are very few targeted therapies for RB. Aptamer-toxin, antibody toxin, Aptamer-doxorubicin, Aptamer-siRNA conjugates using higher expression of Epithelial cell adhesion molecule protein expression on RB cell surface has resulted in targeted nanodrugs in preclinical evaluations of RB therapy. Gold nanoparticles (GNPs) fabricated using green synthesis methods could reduce the side effects associated with chemically synthesised metallic nanoparticles. The GNPs synthesised using *Vitis vinifera* L. extracts were nontoxic to RB cells and when they are conjugated to therapeutic peptides, the metal peptide conjugates exhibited efficacy in killing RB cells. The biologically safe preclinical evaluations should lead to novel therapy options for RB.

Keywords: Retinoblastoma, targeted therapy, green synthesis of nanoparticles

1. Need for targeted drug delivery

Targeted drug delivery is a major thrust area in clinical research [1]. Ligand is the one which carry the drug molecule to the site of action in targeted therapy and needed to avoid the off target effects from the chemotherapy drugs. In general, anti-cancer agents are potentially toxic to human cells. The purpose of the drugs is to eliminate the tumor cells from the afflicted tissue.

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While doing so, the chemotherapeutic drugs will affect the non-tumor cells which is otherwise called off targeting effect. Further, targeted therapy also needed to overcome drug resistant tumor cells [2]. Toxins or drugs when made into Nano formulation, there could be a behavioural change with respect to the drug and its effects. Conjugates such as antibody-based ligands and aptamer-based Nano conjugates do have different effect on the tumor cells. Therefore, these studies have gained importance in recent times as they give therapy options for cancer patients [3].

Several Nanomaterial based drugs are in various stages of clinical trials. The size of nanoparticles is generally below 100nm. However, submicron nanoparticles are also in various stages of clinical trials and waiting for FDA approval. The list of nano formulations waiting for FDA approval are propriety material and hence cannot be listed. The nanoparticles that are usually in the process of development include Liposomes, Nanocrystals, emulsions, iron-polymer complexes, Micelles, Drug polymer complexes, Drug-protein complexes, Dendrimer, polymeric complexes, Nanobubbles, Silica nanoparticles, Drug-lipid complexes, Drug-metal complexes, Protein nanoparticles, Drug nanoparticles, solid lipid nanoparticles, nanotubes, metal-protein complex, metal-nonmetal complexes and metal-polymer complexes, metal-nonmetal complexes and metal-polymeric complexes [4]. However, the first five nanoparticles make up to 85% of the nanoparticles for FDA approval and the agency has used special guidelines for approval of Nanomedicines [5]. Targeted therapy gained widespread importance as conventional chemotherapeutic drugs are associated with systemic toxicity, lower drug response rates, drug resistance and minimal tumor specificity. Monoclonal antibodies against proliferation, angiogenesis and metastasis have become therapeutic options in cancer [6-8]). There is a debate about conventional therapies versus targeted therapies in childhood glioma treatment [9]. Carboplatin and Vincristine drugs that are used to treat Glioma that cause severe neuropathies in children [8]. Precision and personalised medicine is becoming standard care in treating cancer patients. With the recent advances in the targeted therapy, the nanotechnology also had its share in the outcome of targeted therapeutic advances. Linkers and nanodrug conjugates are the constituents of targeted delivery systems and will be discussed further.

2. Cross linkers and its strategy

Three types of crosslinkers are used in binding two reactive molecules as mentioned in the Figure 1. They are homo bifunctional, heterobifunctional and photoreactive crosslinkers. Protein or peptide crosslinking agents contain primary amine, sulfhydryl's, carbonyls and carbohydrate containing moieties. These groups are involved in crosslinking the ligand and drug molecules for targeted therapy. Homobifunctional crosslinker like Di succinimidyl tartarate (DST) combines similar functional group (Primary amine) containing molecules. Heterobifunctional crosslinker combines two different functional groups in the linker and drug, Self-conjugation is minimised in this method. Photo crosslinker uses specific light wavelength to cross link the linker and drug molecules.

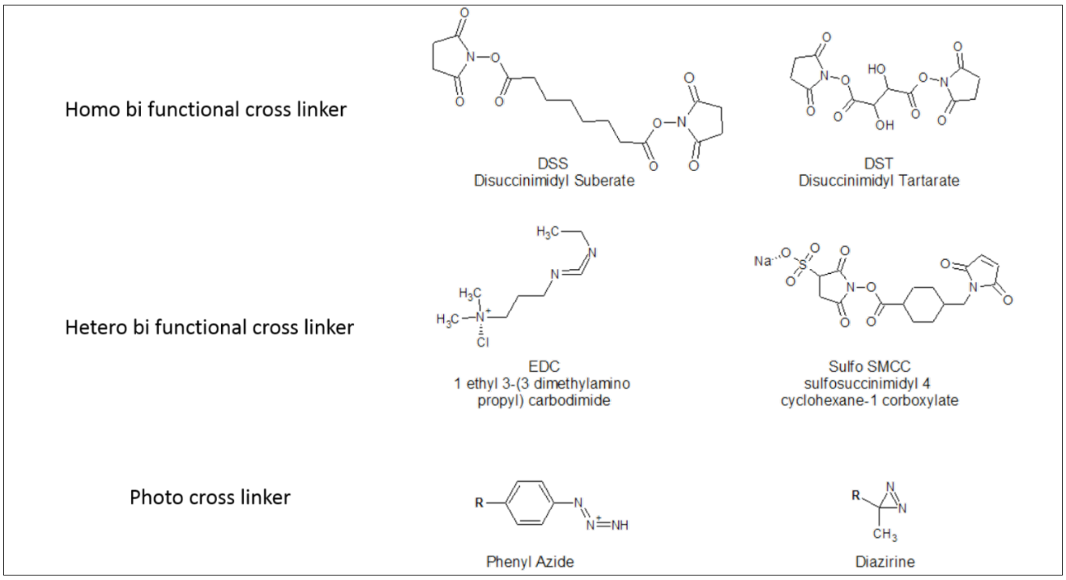

Figure 1.
Types of cross linkers available

3. Polymer nanodrug conjugates

Polymers are very well received by the scientific community due to the less toxicity issues, better renal clearance and biodegradable. Nanoparticle-based drug delivery system, has benefits such as surface to volume ratio, drug bioavailability, biodegradability, drug reactivity. But, the toxicity rising from various parameters such as chemical composition, size, and surface charge needs attention. Nanodrug delivery system involves systemic as well as local drug delivery for a given disease type. The toxicity is less in local delivery than systemic delivery. Several nanodrugs using polymers are in various stages of clinical trials. Examples CT2103 developed by Cell therapeutics is Paclitaxel conjugated to Poly-L Glutamic acid polymer, The bound drug to the polymer is inactive. Therefore, this is less toxic to normal cells. The tumor cells have enhanced permeability because of increased vasculature and lower lymphatic drainage resulting in targeted uptake to the tumor tissue [10]. Poly ethylene glycol (PEG) is another polymer which has been extensively used for different pharmaceuticals. Pegylated doxorubicin has been approved for ovarian/breast cancer in the year 1995 and the product is named as Doxil by M/s. Othobiotech company. Pegylated asparaginase against acute lymphocytic leukaemia has been approved in the year 1994 by Enzon [10]. Polymer such as poly lactic acid gallic acid co polymer (PLGA) had been used to prepare nanoparticles encapsulating antitumor drugs to fight against triple negative breast cancer [11] PLGA is highly biodegradable in human physiology and gets converted into CO_2 and H_2O [12] .

4. Peptide conjugate for targeting tumor cells

Peptide molecules are short chain of amino acids linked via amide bond. These peptides are derivatized to target the cell surface proteins. The peptide ligands can be conjugated to the drug molecules with various linkers. The advantage of peptide sequence is that it can self-assemble to form nanoparticles. The pH responsive polypeptide doxorubicin conjugate for the increased cellular uptake by the tumor cells and to reduce the drug resistance [13]. The particles size between 5 and 200nm are likely to be accumulated in the tumor tissue over the time, as it extravasate in lymphatics canal. The targeting can be passive or active way to the cells. The enhanced permeability and retention (EPR) phenomenon is common in several solid tumors. Longer circulation of active ingredients are important requirements for drug efficacy. Small molecule drug will not be able to achieve longer circulation times but nanomedicine can have prolonged circulation in the blood stream. However, greater attention needs to be given to the stability of the nanoparticles because most of the nanomedicine releases the active drug even before it reaches the tumor site. Hence, conjugation with specific ligand does help in reaching the tumor site exclusively. Active targeting uses receptor mediated cellular uptake using several ligands such as antibody, affibody, nanobody, aptamers, peptide etc. As mentioned earlier, ligands are the molecules that deliver the drug molecules to the site of action. Antibody is large molecule and is immunogenic. Affibodies are smaller version of antibody comprising part of antibody and size ranging in 6.5 kDA [14]. Affibodies are used as ligands targeting cancer for theragnostic [15]. The aptamers are small molecules such as DNA / RNA /peptides which binds the receptor with good affinity. Usually, they are non-immunogenic due to their smaller size [7,16-18]. Interestingly, active targeting reduces the off- targeting effect and improves the accumulation of payloads near the target site. Third way is triggered release on to the target site. The triggering mechanism could be of pH or temperature or specific wavelength guided cleavable linker. Both active and passive targeting relies more on the EPR effect. Thermodox a formulation that releases the drug on elevation of temperature has been developed by Manzoor *et al,* that possessed targeted ability to tumor tissue [19]. Similarly, pH change can be used as a tool to deliver the payload to the tumor cells.

5. Aptamer conjugates

Tumors such as brain liver, lung pancreatic and breast can be targeted through epithelial cell adhesion molecule (EpCAM), Intercellular Adhesion Molecule 1(I-CAM), Neural cell adhesion Molecule (N-CAM), Epidermal growth factor receptor (EGFR) and human epidermal growth factor 2 (HER2) receptor. Targeting cell needs different strategies for different tumor types. Further, reaching the tumor cell possess challenges due to physical and biochemical obstacles.

Aptamers can be short oligonucleotides or amino acids that can be synthesized through systemic evolution of ligands by exponential enrichment (SELEX) procedure against a suitable target protein. They exhibit high sensitivity and specificity (“apt” means “fit” in latin language). The aptamers has been raised against various target proteins of molecules such as CD30, CD28, CD44, CD133, HER1/2, MUC1, EpCAM, and Nucleolin [17]. Aptamers need to be characterized for binding efficacy and specificity. Organic moieties such as aptamers conjugated with different drugs, toxins, for targeting the disease cell types are biodegradable. They being small molecules, may not give toxicity issue but the drug molecule when it gets attached with the ligand may exhibit toxicity [20, 21].

6. Antibody conjugates

Antibody is a well-studied ligand molecule for targeted tumor therapy [3]. This magic bullet invented by a Nobel laureate, Paul Ehrlich a Russian scientist, brought the idea of making conjugate to target specific cell types. While choosing the antibody for conjugate, we need to keep in mind the availability of humanized, fully humanized, and mouse antibodies. The antibody IgG type that is general preferred isotype, due to its ability to trigger, both antibody mediated cytotoxicity and complement dependent cytotoxicity. The mechanism of antibody drug conjugate has steps including cell adhesion, internalization, fusing with endosome, cleavage of the drug from the antibody and causing cell death. This process must be thought when designing antibody drug conjugate. The linker is of two types: cleavable and non-cleavable linkers. Cleavable types are preferred due to its rapid clearance and unaltered functional activity of the released drug. The cleavable linkers are of 3 categories: linkers sensitive to pH, proteolysis, and glutathione oxidation. Proteolysis sensitive linkers are cleaved by cathepsin B in the lysosome. Linkers sensitive to pH change releases the drug from its complex when the pH is altered. However, this type is not generally preferred due to non-specificity. Thiol sensitive linkers are sensitive to glutathione concentration and in general cancer cells have high amount of glutathione and drugs are released into the cancer cells specifically. The drugs choice for the targeted therapy is incredibly challenging due to different characteristics from the drug itself targeting different cellular functions [22].

7. Toxin conjugates

Toxin conjugates are similar to antibody drug conjugate. The only difference is the property of the ligand. In this case, toxins are generally derived from microbes [23], plants [24] and animals [25]. These toxins possess activity from its structural features. The possibility of losing their activity is higher when conjugated to ligand. The ligand functional efficacy such as DNA damaging or microtubule toxicity should be assessed initially before undertaking drug efficacy at the tumor site. Again, if the ligand is oligonucleotides, the stability of the molecule in the presence of toxin needs to be assessed [26]. It is also necessary to study the stability of the ligand itself in the circulation. If it is oligonucleotides, substitutions of 2-OH group with 2’ Fluoro, 2-Amino or 2-O Methoxy need to be done for the stability against the endogenous nucleases. The bacterial toxins are classified into several types based on their functions: like pore forming toxins, single chain toxin and re-routing or transporter toxin [27]. Single chain toxins are very potent and interfere with several cellular processes and metabolisms. Diphtheria toxin a known single chain toxin, interferes with ribosomal translation process. Another single chain toxin, exotoxin from *Pseudomonas aeruginosa*, which also shares similar structure to diphtheria toxin, but uses different mechanism of toxicity [28].

8. Drug conjugates

Drugs conjugated with its ligands such as aptamer, antibody, affibody brings effective targeting of tumors. The drug efficacy depends on the payload and the ratio between drug and ligand. The polarity of drug, linker and ligand needs to be synchronized for making a stable conjugate. Otherwise, conjugate cannot be stable in systemic circulation. Several reports are published emphasizing various factors during conjugate synthesis. These include: 1. choosing the target molecule on the tumor cells, finding the expression of target proteins and right isotype of IgG, 2. Method of synthesizing the conjugate, and its pharmacokinetics in the system, PK PD modelling and determinations for the conjugate, 3. Suitability of the method for large scale preparation, and possible resistance development from the drug conjugate. All these factors need to be checked before preparing the conjugate [29].

Various targeting methods are described earlier and decision to choose the ligands or the suitable method depends on the type of tumor cells to target. Molecules like Polymer, peptide, aptamer, antibody, affibody, drug, and toxin conjugates have different mode of cellular uptake. Aptamer, antibody and affibody in general go through receptor mediated cellular uptake. Peptide conjugates go through either endocytic machinery or direct cellular membrane transloca-

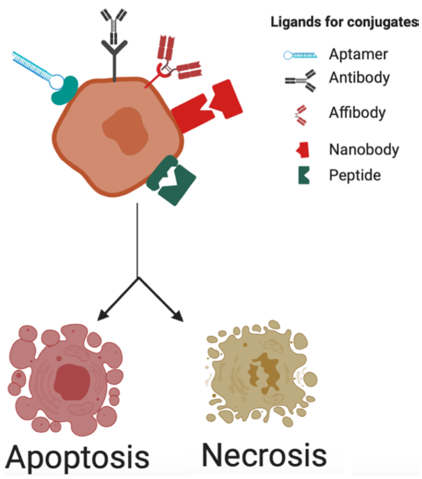

Figure 2.
Schematic representation of different conjugates and its effect on tumor cells

tion. The efficacy of targeted cellular therapy depends on the ligand type and conjugate type. All these conjugates targeting tumor cells can be prepared in green method in the laboratory. The conjugates can bring tumour cell death through the process of apoptosis and/or necrosis (Figure 2). Examples of conjugates and their efficacies towards solid tumours are presented in Table 1.

Table 1.
Efficacy of toxin conjugates against cancer therapy

Types of conjugate	Example and efficacy against Cancer type	References
Polymer drug conjugate	Polyglumex-Paclitaxel advanced lung cancer	[30]
Peptide conjugate	HER2 targeting peptide with TGX-221 (phosphoinositide 3-kinase β inhibitor) against prostate cancer	[31, 32]
Aptamer Conjugate	E3 RNA aptamer- monomethyl auristatin E Conjugate against prostatę cancer EpCAM RNA aptamer – Neocarzinostatin against retinoblastoma	[21, 33]
Antibody conjugate	Anti-EpCAM antibody amanitin conjugate against pancreatic cancer Anti EpCAM antibody saporin conjugate against retinoblastoma	[34, 35]
Drug conjugate,	LGR5 receptor drug conjugate against gastrointestinal cancer	[36]
Toxin Conjugate	RNA aptamer: gelonin conjugate against prostatę cancer	[37]

9. Ocular Drug delivery is challenging and needs Nanotechnology methods

Ocular Drug delivery administration includes topically to the surface of the eye injection/implants, directly into the vitreous (intravitreal injection/implant), or oral method. The challenge includes several barriers to the interior eye such as corneal barrier, scleral barrier, Blood retinal barrier, tear film barrier and vitreous barrier as mentioned in Figure 3. There is need for sustained drug delivery and several Polymers are in use for nano formulations. Several implants are in clinical use for ocular diseases [38]. However, nano formulations for Ocular tumors is still an evolving field. Several tumours are reported from various parts of the eye (Figure 3). The predominant Ocular tumors are, Retinoblastoma in children and Uveal Melanoma in adults, basal cell carcinomas of eyelids, ocular surface squamous malignant neoplasia and ocular adnexal lymphomas are other ocular tumors [39]). The advances in therapy using polymers, toxins, aptamers and metal nanoparticles for ocular tumors is discussed [4]. However, nanoparticles as therapeutic targeted vehicles which were synthesised using green synthetic methods are examined below.

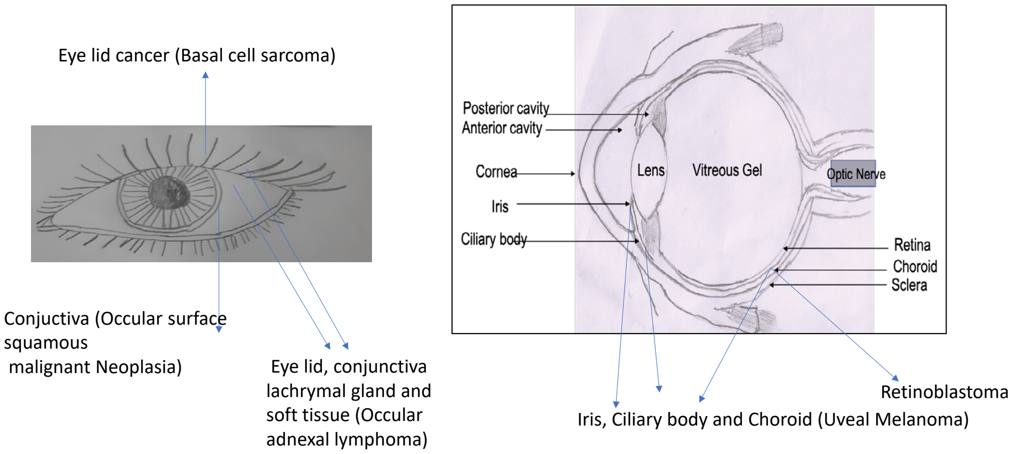

Figure 3.
Schematic of internal structures of Eye and Eye cancers

10. Retinoblastoma is intra ocular tumor that effects children

Retinoblastoma effects children mostly below 5 years and occurs 1 in 15 000–20 000 births. The tumor progression could be due to genetic and sporadic mutation in RB gene. RB gene is tumor suppressor molecule that is involved in cell cycle progression. Loss of RB leads to uncontrolled cell division through activation of elongation factors [39]. A small number of tumors (3%) are caused due to MYCN amplification [40]. Additionally, the children are affected by secondary tumors of brain, lung and sarcomas, with poor prognosis [41]. The tumors are usually identified at higher grades in developing countries and generally, enucleation is prioritised to save the child's life followed by preserving vision, and lastly to minimize any side effects of treatment. Vincristine, Topotecan, Carboplatin and Etoposide are used presently in clinical practise, which may have side effects in children and targeted therapy is needed to reduce off target effects.

11. Effect of ligand and toxin on RB tumor cells

Recently, aptamer Neocarzinostatin toxin conjugate was synthesized to target epithelial tumor cells. Neocarzinostatin is a DNA damaging agent isolated from an organism *Streptomyces carzinostaticus*. The drug molecule was failed in the clinical trials performed in Japanese population cohort 4 decades ago [42]. The reason for its failure was investigated and found that the immune response from the patients are features for its failure. Aptamers are less immunogenic than antibody and hence can be conjugated with the toxin to reduce the side effects. Biophysical experiments proved the interactions between Neocarzinostatin and EpCAM aptamers are not damaging the aptamers [26]. The aromatic amino acids of Neocarzinostatin interacted with certain RNA molecules of the aptamer residues and exhibited a stable complex. When aptamer and toxin were conjugated, the aptamer was found to be stable *in vitro*. The effect of conjugate was then studied against breast cancer and Retinoblastoma cells. Surprisingly, the global gene expression profiling using DNA micro array results showed that native drug alone could induce necroptosis in the retinoblastoma tumor cells [21]. Further, the pathway that is triggered when native neocarzinostatin and the aptamer conjugate were used on the cancer cells, is necroptosis. In another study saporin toxin molecule which is ribosomal inactivating agent extracted from *Saponaria officin*alis plant, was tested on RB cells. This saporin internalized to the cells through receptor mediated endocytosis. This toxin could penetrate the epithelial cells only when conjugated with EpCAM antibody. The cells that does not take conjugate is not get affected by the saporin toxicity unlike neocarzinostatin. Hence, in saporin antibody conjugate showed clear distinction between native saporin and conjugate suggesting the significant reduction in off- targeting effect [35].

12. EpCAM mediated targeted delivery in Retinoblastoma preclinical models

The EpCAM aptamer was conjugated to Doxorubicin and this conjugate selectively killed Retinoblastoma cells sparing the normal cells [43]. Further, when the aptamer was conjugated to siRNA of Oncogenes such as PLK1, BCL2 and STAT2, the aptamer conjugate could selectively kill cancer cells and spare normal cells. The efficacy of the aptamer depended on the magnitude of cell surface EpCAM receptor expression. The higher the surface receptor expression, Ex NCC RBC 51 cells with elevated receptor levels, efficiency of killing the Retinoblastoma cells is higher. Further, WERI cells with lower EpCAM receptor expression resulted in lowered cancer cell death compared to NCC RBC 51 cells [44]. The aptamer conjugates also reduced the tumor burden in the xenograft model of Retinoblastoma [45]. Further, the molecular mechanism underlying the PLK1 si-EpCAM aptamer conjugate, studied by global phosphoproteomics analysis identified signalling mechanism mediated by P53, tumor suppressor protein. Heat shock proteins and Hypoxia factors are dysregulated in several cancers [46].

13. Metal nanoparticles for drug conjugations in Retinoblastoma

Metal nanoparticles bears no concern if it is being considered for *invitro* diagnostics. Because metal clusters previously were not advisable due to its systemic clearance issues [47]. However, recently several inorganic nanoparticles containing iron, gold, silver and Gadolinium are in various stages of clinical trials for various cancer diagnostics and therapy[48][4]. Gold nanoparticles (GNPs) have gained more attention because of their biocompatibility and potential applications in various sectors [49]. Efficacy was observed after conjugating to PEG and recombinant tumor necrosis factor (CYT-6091) in Phase I clinical trials [50]. Colloidal gold AuroLase® was used in a pilot study with 85% efficacy in small set of prostate cancer patients [51]. Environmental friendly approach avoiding toxic chemicals for GNP preparation gained importance recently. Several plant, fungal and microbial extracts were used for GNP fabrication. GNP was fabricated by rapid simple method using phytochemicals from fruits of Vitis. vinifera L. The Sodium tetrachloroaurate was reduced to Au by the polyphenols instead of using toxic Sodium borohydride for the synthesis of metallic nano-particles [52]. These GNPs could be used as SERS nanotags and can be used for bioimaging tumors. Green synthesized GNPs were not toxic to RB cells. An Oncogene, HDM2 that is upregulated in RB tumors was chosen to target RB tumor cells. A therapeutic peptide that targets this Oncoprotein is conjugated to GNPs synthesized from grapes [53]. The peptide is called HDM2 that it interferes with the interaction of HDM2 protein to P53 tumor suppressor protein. This GNP-Peptide conjugate was effective in reducing tumor volume and growth in mouse xenograft models of RB tumors. HDM2 peptide primarily functions by increasing the stability of P53 tumor suppressor protein [54]. The transcript levels of P53 was significantly increased in GNP, GNP-HDM2 treated compared to the untreated control. The P53 increase caused rise in the process of programmed cell death and apoptosis markers increased in RB tumor model.

The GNPs synthesized by *Vitis vinifera* L. was conjugated t*o* Peptide A an antioxidant peptide (Pep-A) to make nanoconjugate that has free radical scavenging activity[55]. The antioxidant properties associated with the natural polyphenols of grapes and peptide A could have enhanced free radical scavenging property. The GNPs-Pep-A nanoparticles were more efficient in scavenging reactive oxygen species than either GNPs or Pep-A, indicating a synergistic antioxidant effect in the bioconjugate. These GNP-Pep A were efficient therapeutic molecules for eliciting RB cell death. Thus, GNPs conjugated to different peptides; HDM2 peptide and Pep A were more efficient as

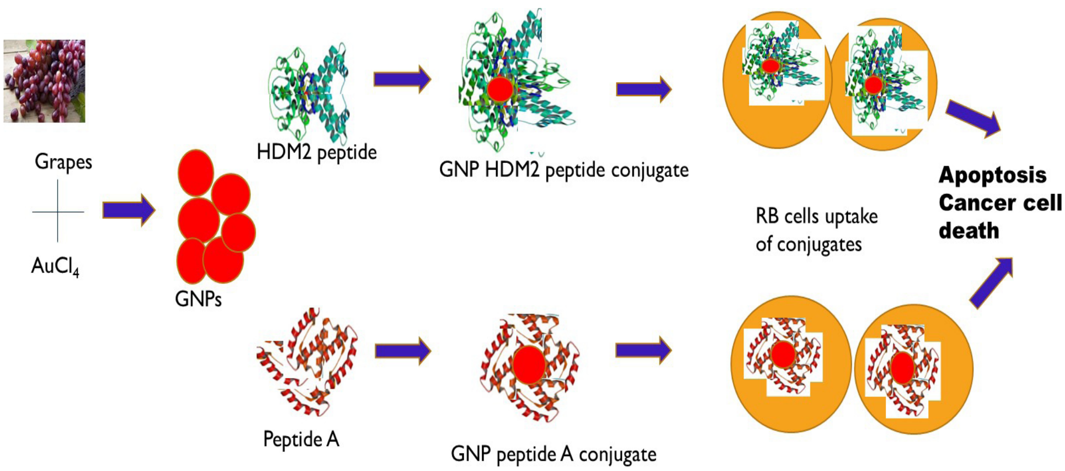

Figure 4.
Schematic representation of Green GNP-Peptide conjugates for RB cell therapy.

therapy molecules compared to the GNPs alone indicating that metal nanoparticle peptide conjugates as mentioned in Figure 4 are more apt for Retinoblastoma therapy in the *invitro* preclinical evaluations.

In conclusion organic molecules such as target protein, ligand and drug/ toxin are the only three components involved in targeted therapy. The target tissue location, Immunogenicity of ligands and efficacy of the drug or the toxin are factors governing them. Target tissue location takes primary lead due to its variation in payload distribution through the tissue types. Intra tumoral always has better drug availability than systemic delivery. Systemic delivery holds the promise for the interior tissue and inaccessible sites. Hence, target site identification is a mandate before synthesizing ligands. Then immunogenicity of the ligands depends on the host response. To reduce the immunogenicity, ligands such as aptamer, affibody, peptide, nanobody which are less immunogenic than the antibodies are preferred. Toxins, chemotherapy drugs and therapeutic siRNA could be delivered to Retinoblastoma tumors in the preclinical evaluations in future to reduce off target effects. Among the metallic nanoparticles, GNPs synthesised using grapes rich in anticancer and antioxidant properties could be a valuable alternative.

14. Perspective and future scope

Pharmacology and therapeutics have found a niche in the recent decade due to the development of nanotechnology. The pitfalls of pharmacology such as toxic side effects from the drug, off- targeting effect from the drug, over dosage, solubility and stability issues of pharmaceutical drugs have been addressed using nanomedicine approach. Green synthesis of pharmaceuticals had strengthened the niche further. However, there are lacunae that needs more attention in the future such as scalability, reproducibility, cost factor enabling synthesis of drug conjugates for targeting various diseases.

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A Xenobiotic Pollutant Detection for a Zero Pollution Environment

Abstract: In general, xenobiotic pollutant describes a man made chemical compound that are foreign to environment (not naturally produced) and expected to be present within the organism. Morpholine (1-oxa-4-azacyclohexane) is one such chemical which is extensively used in myriad of industries like in rubber industry (vulcanization accelerator), plating industry (corrosion control), textile industry (lyocell process), personal care industry (formulation of face wash), synthesis of a large number of drugs, crop protection agents and optical brightener agent. Consequent to its wide range of industrial applications and high solubility, significant amount of this chemical is released via different industrial effluents into the environment. In the natural environment, its secondary amine functionally undergoes a typical chemical transformation reaction called nitrosation to form a carcinogenic product namely N-nitrosomorpholine. The large-scale annual usage of morpholine and its potential carcinogenic effects thus have an environmental interest for its detection in natural as well as in industrial effluents. In this article, the focus of research is to develop an analytical technique, which should be efficient, effective, sensitive and feasible for estimating minute ranges of this analyte. Herewith, a spectrophotometric based microassay or die away test for the quantification of morpholine have been developed and validated. Thus with the help of this micro-assay, a directly correlate the morpholine content with the color intensity of the product formed in the above assay is established with a regression correlation coefficient of 0.99 and sensitivity of 2–10 ppm. This simple cost effective method of detection of morpholine at trace level could set a stage for the early detection of frequently faced environmental contaminants which if undetected may be life threatening.

Keywords: Morpholine, N-Nitrosomorpholine, Gas chromatography, Die-Away test

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1. Introduction

We are faced with problem of anthropogenic activity and production of either amounts or types of chemicals that are not natural and easily enter into the biosphere. These chemicals therefore called as xenobiotics (greek *xenos* = strange, foreign, foreigner) and is one of the sources of manmade pollutants (xenobiotic pollutant) to environment. The total annual world production of synthetic organic chemicals is over 300 million tons [1] with more than 1000 new compounds fabricated every year, so that the actual diversity of chemicals reaches approximately 20 million of substances. Depending on their fate in air, water and soil, xenobiotic pollutants may become available to microorganisms to higher tropic level of biota and may persist or behave as recalcitrant for months to years in different environmental compartments or levels. Due to unnatural structural feature of xenobiotic pollutant, it does not necessarily imply that xenobiotics are harmful/toxic to living organisms, but it may undergo a series of chemical modification or transformation reactions through different routes either in the environment or in the biological system to make it deteriorate and pro-carcinogen or teratogen (causing birth defects). Specific to morpholine is that morpholine itself is neither a carcinogen nor a teratogen, however, the moment it releases into the environment through different industrial effluents, it undergoes a typical chemical transformation (Figure 1) called "Nitrosation" (in presence of aqueous solutions of nitrites or by gaseous nitrogen oxides, e.g., N_2O_3, N_2O_4) and converts into N-nitrosomorpholine (NNMOR) [2–5].

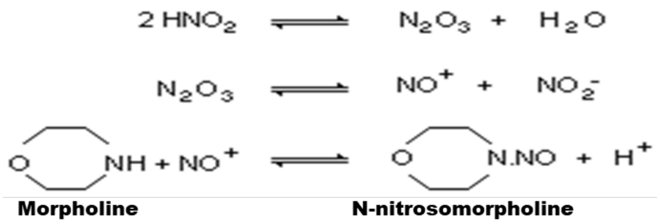

Figure 1.
Nitrosation pathway of morpholine

In addition, NNMOR is known to act as a mediator for various debilitating cancers (carcinogen) associated with organs like digestive tract, respiratory tract, kidney and liver. Simultaneous exposure of laboratory animals to morpholine and nitrites has led to number of different cancers [6].

1.1. Xenobiotic pollutant morpholine: availability, properties and utilities

Morpholine (C_4H_9NO) is an extremely versatile synthetic organic compound. Chemically it is six-membered heterocyclic compound that features amine and ether functionalities (Figure 2). It is designated as volatile organic compound (VOC) and commonly used various in organic synthesis.

Table 1.
Typical physical, chemical, and biological properties of morpholine and its different industrial applications

Morpholine	Feature	References
Physical	Liquid, Colorless, Oily	[8]
	Odor: Amine like	
	Flammable, Corrosive	[9]
	Hygroscopic, Volatile Sensitive to moisture	[10]
	Density: 1.007g/cm^3	
	RI: 1.4548 at 20°C	[11]
	MP: -5°C and BP: 129°C	
	VP: 6mmHg, VD: 3 g/cm^3	[12]
	FP: 31°C	
	Specific gravity: 1	
	AIT: 275°C	
	Purity: 99–100% (contaminated with N-ethylmorpholine and ethylenediamine)	[13]
	Storage: in cool dark place Stability: Room temperature Morpholine should be protected from atmospheric moisture and carbon dioxide	[14, 15]
Chemical	Molecular formula: C_4H_9NO	[16]
	Molecular wt: 87.12	
	2° Amine	
	Synonyms: 1-oxa-4-azacyclohexane; Diethylene oximide; Diethyleneimide oxide	
	Solubility: Miscible in water and other solvent ethanol, benzene, and acetone	[17]
	Acidity (pKa): 8.36; Strong Base, Polar; pH: 9.4 (0.01% w/w)	[18]
	Reactivity: Moderate but violently with O.A	[12]
Biological	Health issue: Serious	[19]
	LD $_{50}$ (Oral): 525 ppm in mouse LC$_{50}$: 1.32 ppm in mouse 2 Hrs.	[12, 20, 21, 22]
	PEL-TWA: 20 ppm	[23]
	IDLH: 1400 ppm; REL-TWA: 20 ppm	[24]
	Antibacterial and Antimycotic properties 0.5% and 10% morpholine inhibits the growth of a variety of pathogenic bacteria and mycosis respectively	[14, 25]

Table 1. cont.

Morpholine	Feature		References
Biological	Blue vision, grey vision or haloes, “glaucopsia” Corneal oedema with “hazy vision” and halo phenomena around lights		[26, 27, 28]
	The substance has toxic properties which affects digestive, respiratory, and cutaneous systems Harmful if swallowed Toxic in contact with skin, irritating to eyes, nose and throat Causes severe skin burns and eye damage and necrosis Toxic if inhaled and causes nausea, headache, or difficult breathing Suspected of causing genetic defects Causes damage to organs through prolonged or repeated use		[28, 29, 30, 31]
	Harmful to aquatic life Damage to living resources BOD: 0.9%, 5 days; 5.1%, 20 days		[16]
Major industrial application	Metal industry	As an anti-corrosion and anti-rust-proof factor in the protection of various metals It is a strong alkaline that rapidly absorbs humidity and evaporates into the air	[32]
	Personal care industry	In cosmetics as an additive to hair dye, conditioners, and shampoos	[33]
	Agriculture industry	To preserve fruits for a longer time and to process food, morpholine is used in waxes as wrapping coatings	[21]
		Morpholine and its salts have been used as components of protective coatings applied on fruits and vegetables	[34, 35]
		As a pesticide, bactericide, herbicide, and fungicide	[36]
	Paint industry	As a good solvent for paints and resins in the paint industry It is a cheap and readily available alkaline catalyst	[37, 38, 39]
	Pharmaceutical industry	As a catalysts, antioxidants, bactericides, analgesics, anesthetics, and other physiologically active agents	[8]
	Textile industry	To dissolve cellulose for the manufacturing manmade fibers	[40]
	Other industry	Solvent for resins, waxes, casein, and dyes	[41]
		Tobacco industry (cigarette), refinery, power plants (additive in stream system to adjust the pH levels in water), rubber industry (vulcanization accelerator) and paper industry (optical brightener)	

Where: MP: melting point, BP: boiling point, VP: vapor pressure, VD: vapor density, FP: flash point, RI: Refractive Index, AIT: auto ignition temperature, LD: lethal dose, LC: lethal concentration, PEL: permissible exposure limit, REL: recommended exposure limit, TWA: time weighted average, IDLH: immediate danger limit for health, OA: oxidizing agents, BOD: biological oxygen demand, OSHA: occupational safety and health administration, NIOSH: national institute of occupational safety and health

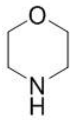

Figure 2.
Chemical structure of morpholine where O, N and H indicate the Oxygen, Nitrogen and Hydrogen respectively

The major physical, chemical, biological properties of morpholine and its versatile applications are summarized in Table 1. However, the typical uses of morpholine are rubber accelerator, 40%; corrosion inhibitors, 30%; waxes and polishes, 5%; optical brighteners, 5%; and miscellaneous, 20% [7] (Figure 3).

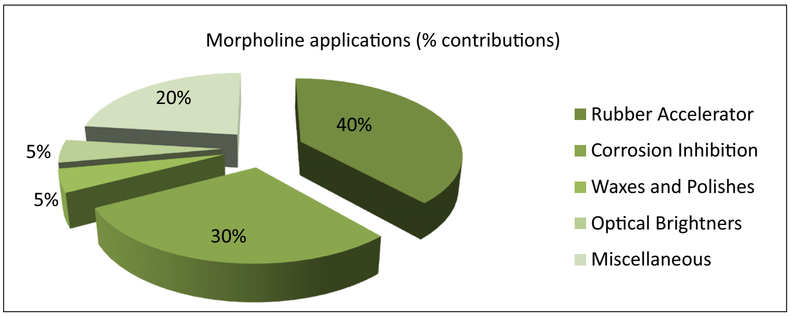

Figure 3.
Percentage contribution of moprpholine applications in different industries

1.2. Different methods of quantification of morpholine or its derivatives

The methods available for estimation of morpholine involve chemical reactions that are characteristic of the individual functional groups. Many less sensitive and indirect methods based on titration [42] and chemical conversion to oxides- chemical oxygen demand (COD) [43] have been used in the past 2–3 decades. Several other methods based on analytical instruments such as capillary isotachophoresis [44, 45] (Figure 4), gas chromatography (GC) [16, 46, 47, 48] (Figure 5a, 5b and 5c) high pressure liquid chromatography (HPLC) [49, 50, 51] (Figure 6a, 6b and 6c), ion/cation exchange chromatography [52, 53, 54] (Figure 7a and 7b) are in use for quantification of morpholine. Each analytical method has its own advantage and limitation (Table 2), e.g. cation-exchanger resins have a high capacity for amines, but they do not work well in the presence of electrolytes e.g., NaCl, Na_2SO_4 [55]. Morpholine determined by gas chromatography (Figure 4a, 4b, and 4c) require packed as well as capillary columns with detectors as either flame ionization detector (FID), flame photometric detector (FPD), nitrogen selective detector (NSD), mass spectrometry (MS) or thermal energy analyser (TEA). However, in HPLC based estimation, it needs ultraviolet (UV), TEA, and refractive index (RI) detector and derivatization is required in same analytical setup of condition (Table 2). These methods require sophisticated, expensive analytical instruments along with specific sample preparation steps and provide an opportunity to rapidly evaluate effluent composition/treatment methods for estimation of morpholine adopted in industrial settings.

Table 2.
Different analytical methods of estimation of morpholine or its derivatives

Analytical method	Characteristic feature	Detection limit	Specificity	Remark	Reference
Titration	Standard procedure of Karl Fischer titration	mg/L range	Morpholine	Titrated in acidified reagent solutions (Benzoic acid, EDTA, amine N-oxide) to a stable end point	[42]
COD	The amount of a specified oxidant that reacts with the sample under controlled conditions	230 mg/L = = 423 ppm COD	Morpholine	The specific COD for the concentration of morpholine is 1.84 mg/L	[43]
Capillary isotachophoresis (ITP)	Identification of the analytes are based on the corresponding step height	30 mg/L	Morpholine and its derivatives	The separation capillary is filled with a discontinuous buffer: one the leading electrolyte and the other the terminating electrolyte	[44, 45] Figure 4
GC-FID	An amine specific column and flame Ionization detector with the split mode injection	μg/L to mg/L (0.001– –1000 ppm)	Morpholine, Methyl morpholine	Amines tend to be strongly adsorbed and decomposed on the columns and give tailing and poor reproducibility	[46, 47, 48] Figure 5a, Figure5b
GC-MS (Electron ionization)	Analyze morpholine on this basis of generation of ion followed by its separation due to differences in m/z	mg/L	Morpholine	Positive mode major m/z peaks: 56, 57 and 87	[16] Figure 5c
HPLC	Analytical column of Supelco-sil-ODS with an isocratic mobile phase and UV detection at 280 nm	0.01 to 10 μg/mL	Morpholine and its derivative	It has been derivatised with N-succinimidyl-p- -nitrophenylacetate	[49, 50, 51] Figure 6a, Figure 6b, Figure 6c
Ion/Cation exchange ion chromatography (IC)	IC with Dionex IonPac CG19 guard column along with suppressed conductivity detection Eluent: 7.5 mM Methane sulfonic Acid	μg/L	Morpholine	Flow rate: 0.25 mL/min Injection volume: 100 μL Concentrator column Dionex IonPac TCC-ULP1	[52,53,54] Figure 7a, Figure 7b

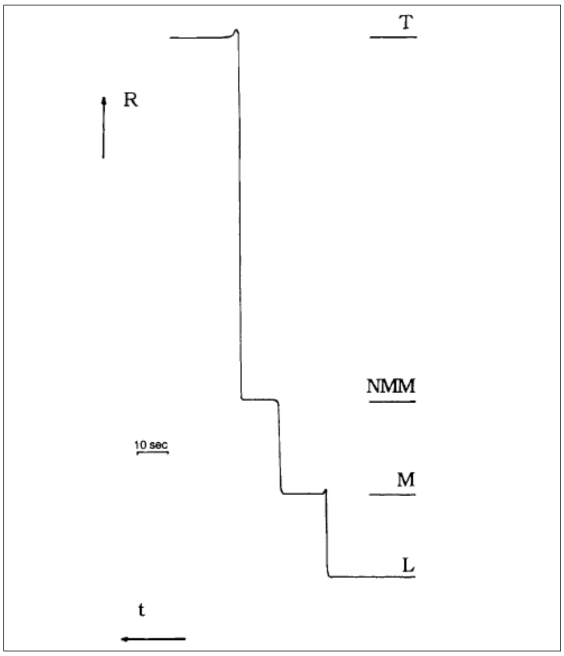

Figure 4.
Isotactophoresis of mixture of morpholine (M) and N-Methyl Morpholine (NMM). L indicates the leading ion, T indicates the terminating ion, R indicates the resistance and t indicates the time in minutes

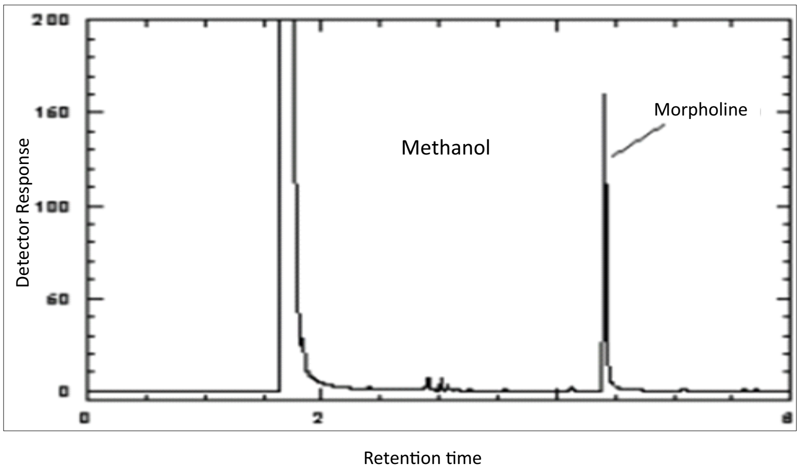

Figure 5a.
GC-FID analysis of morpholine

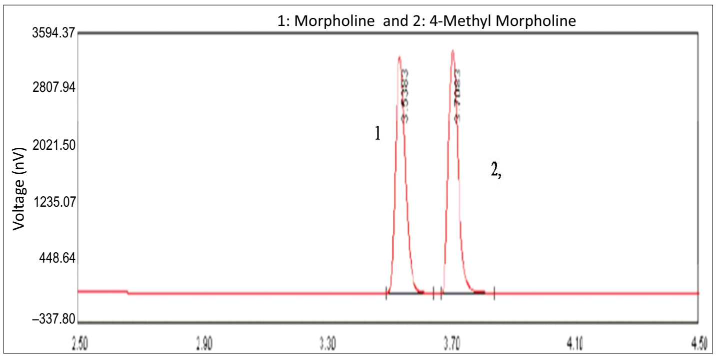

Figure 5b.
GC-FID Analysis of N-methyl morpholine and morpholine;
where 1 and 2 represent morpholine and methyl morpholine respectively

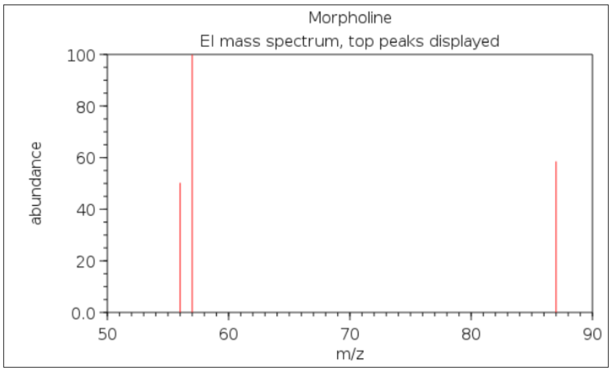

Figure 5c.
GC-MS (Electron ionization) analysis of morpholine where major m/z peaks are 56, 57 and 87

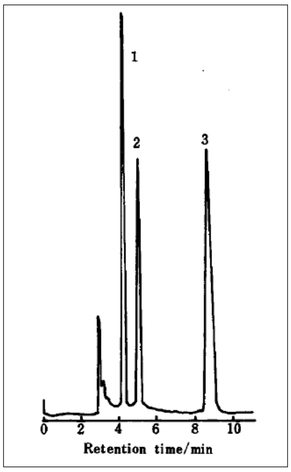

Figure 6a.
HPLC analysis of 1, 2-Naphthoquinone (NQ) derivatized products;
Where Peak 1 = Morpholine-NQ, 2 = Pyrrolidine-NQ and 3 = Piperidine-NQ

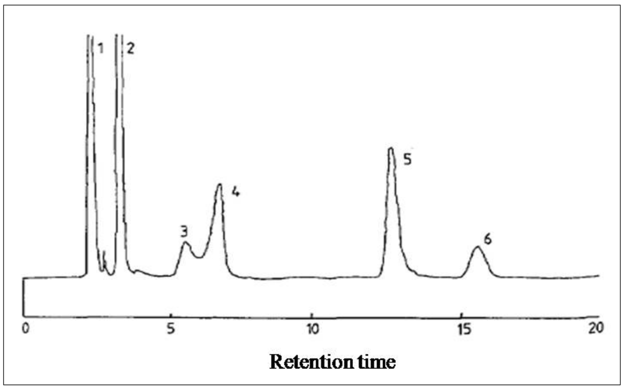

Figure 6b.
HPLC analysis of N-succinimidyl-p-nitrophenylacetate (SNPA) derivatized products. Peak 1 = p-nitrophenylacetic acid, 2 = N-hydroxysuccinimide, 3 = Ethanolamine, 4 = Ammonia, 5 = Morpholine and 6 = 2(2-aminoethoxy) ethanol

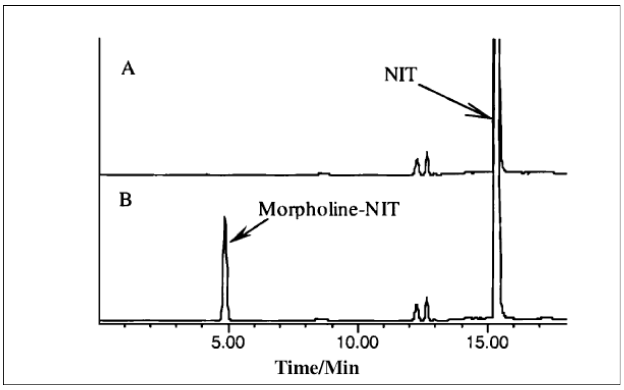

Figure 6c.
HPLC analysis of 1-naphthylisothiocyanate (NIT) derivatized products.
A indicates the filter blank and B indicates the filter spiked with morpholine

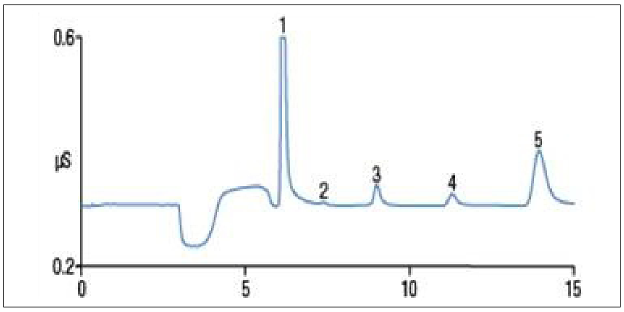

Figure 7a.
Ion chromatography of morpholine (20 µg/l morpholine in 10% methanol) using Dionex Ion Pac CS19 column.
Peak 1 = Ammonium salt, Peak 2 = Potassium, peak 3 = Morpholine, peak 4 = Magnesium and peak 5 = Calcium

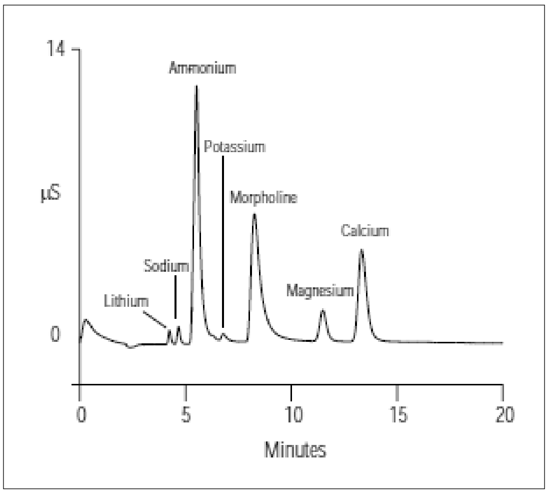

Figure 7b.
Ion chromatography of morpholine mix sample using Dionex Chromatography system

1.3. Development of spectrophotometric estimation of morpholine (Die Away Test)

This study has been designed and miniaturized based on the findings published by WH Stevens and Kirsten Skov. 1965, Giti Emtiazi et al., 2001, and Kumar Rupak and Kapur Suman 2016 [56, 57, 58]. It involves a simple chemical reaction in which morpholine reacts with sodium salt of 1,2-naphtoquinone-4-sulphonic acid (NPQ/NQS) under alkaline conditions at room temperature to form a red/orange coloured product named 4-(4-morpholinyl)naphthalene-1,2-dione which was detected using colour reading machine, the UV – visible spectrophotometer (at 480 nm). The reaction was completed in a span of 20 minutes and since it is light sensitive it was carried out in the dark. Thus with the help of this micro-assay, a direct correlate the morpholine concentration with the colour intensity of the product formed in the above assay reaction is established.

2. Material and methods

2.1. Instrument

An Elisa reader (ELX50/8MS BioTek India) provided with matched wavelength and light controller was used to quantify concentration of morpholine.

2.2. Reagents and Solutions

All chemicals used were of analytical grade. Double distilled water was used for dilution and blanking purpose. Freshly prepared 0.115M (3%) of NQS/NPQ and 1N sodium hydroxide (NaOH) is used for experimental purpose.

2.3. Standard Solution

A standard solution of 1000 ppm (0.1% v/v) of morpholine wasprepared separately and further diluted 1:10 by dissolving 200 µl of 0.1% in 2.0 ml of double distilled water in a micro centrifuge tube. Thus the working solution concentration is 100 ppm (0.01%). The working solution was further diluted to get a series of standard solutions concentration (2–10 ppm) with double distilled water to make a final volume 300 µl (Table 3).

Table 3.
Preparation of calibration curve of standard solution

Calibration level	Final standard concentration [ppm]	Volume of working standard added [µl]	Volume of solvent [µl]
1	2	6	294
	4	12	288
	8	24	276
	10	30	270

2.4. Methodology

Morpholine has been analyzed spectrophotometrically. Suitable aliquots (300 µl) of morpholine working standard solution and unknown sample were pipetted out into standard 96 well elisa plates. 2 µl of 1 N NaOH and 2 µl of 3% of NQS reagent were added to each of well. The solutions were left for 20 min at room temperature in the dark. The absorbance of these solutions was measured at 480 nm against a reagent blank (Figure 8). A calibration plot was constructed by plotting absorbance against different concentrations. Using the calibration curve, the concentration of morpholine was calculated in test sample (in duplicate).

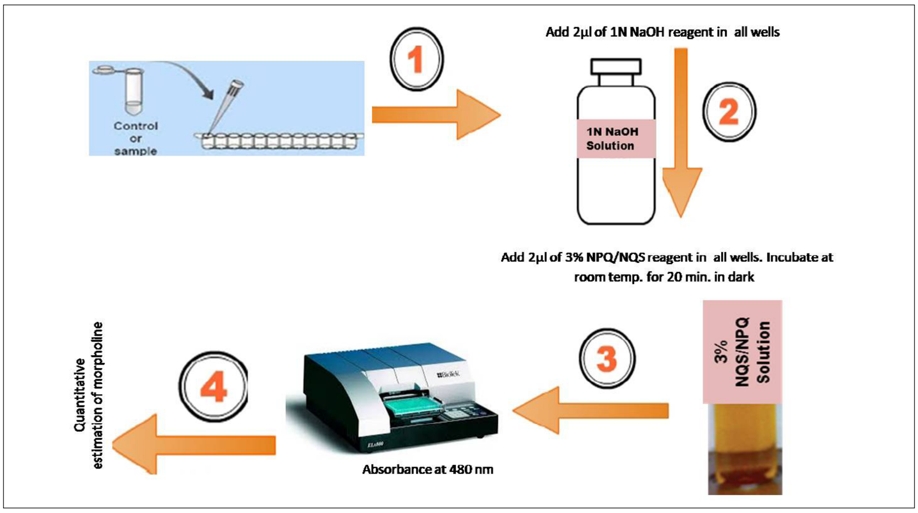

Figure 8.
Brief discription of various steps involved in micro-assay for morpholine detection

3. Result

A calibration curve has been plotted against the concentration of morpholine and the net absorbance (at 480 nm). The concentration of morpholine in test sample was calculated using this calibration curve (Table 4). An excellent linear relationship was existed between the absorbance and the concentration of morpholine (r^2 = 0.997, slope of 0.069, Figure 9). In the subsequent diluted of test sample (1:100), the available morpholine concentration using that standard curve against reagent blank was found to be 104 ppm.

Table 4.
Spectroscopic estimation of morpholine available in test sample

Well No.	Name	Net absorbance	Calculated concentration [ppm]
1	Water blank	0	
2	Reagent blank	0	
3	2 ppm	0.147	
4	4 ppm	0.297	
5	8 ppm	0.562	
6	10 ppm	0.673	
7	Test sample (1:100)	0.072	1.043 × 100 = 104.3

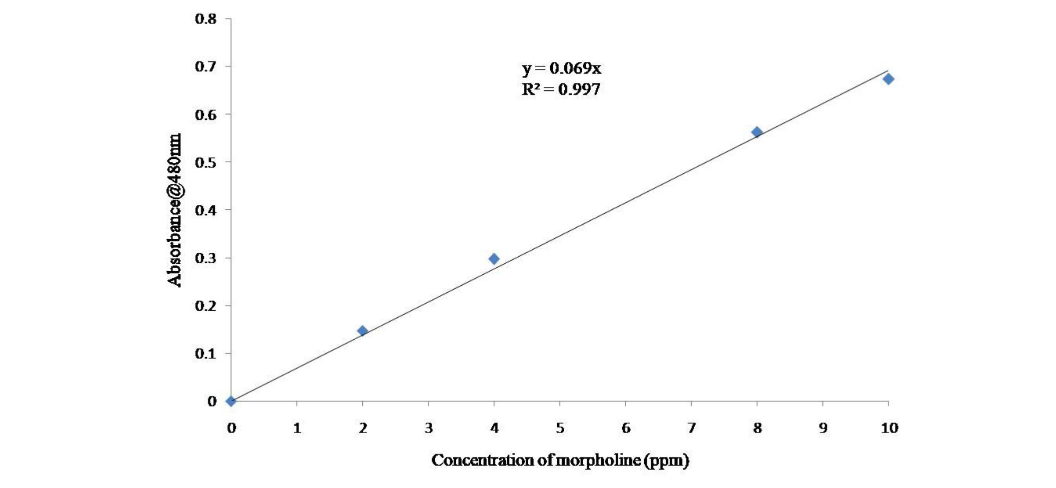

Figure 9.
Standard calibration curve of morpholine

4. Discussion

Although, there are several analytical techniques available for the successful quantification of morpholine content, this approach for which our team had opted was simple, time-effective, and feasible. Also, it doesn't require any sophisticated instrumentation, infrastructure and skill set. The USP of the assay developed require minimal reagent (only 2 common reagents), sample volume, and efforts to estimate the presence of morpholine in a concentration of 2-10 ppm range at a lab cost of < 0.015 USD ($) only. Thus the assay described aforesaid is a small volume, sensitive, rapid, reliable and economical and has a potential to enable it to handle large number of samples if automation can be featured in a device based on this principal of estimation. Further, based on this microassay, we are starving to develop a point of detection (POD) device to estimate the presence of morpholine not in industrial effluents samples but also in other biological samples like glazed fruits and vegetables (data not shown, beyond the scope of the said manuscript) at ppm lavel. Along with addressing the environmental impacts of pollutants, it revolutionize the market presence when consumers around the globe demand for fruits/vegetables of high-quality with its naturality and this proposed device easily indicate that the available fruits/vegetables are either natural or glazed one**.**

5. Conclusions

Based on the nature of xenobiotic pollutant and its health risk, the type and severity of symptoms of morpholine toxicity may vary and truly depends upon the concentration of morpholine. Keeping this in view, there is an urgent need to find out the minimal concentration of xenobiotic pollutant in the environment and the nature of its exposure. To quantitatively assess the minimal concentration of morpholine, a micro assay has been developed and validated. We hope, this efficient and effective micro assay could occupy an iconic position in the field of applied science and set a stage for the development of POD device which aid in the early detection of environmental contaminants so that progression of health threatening effects can be substantially reduced and look forward towards a sustainable solution (SS).

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