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Lemna minor test for characterization of paper mills effluent

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit, abgesehen von den in ihr ausdrücklich genannten Hilfen, selbständig verfasst habe.

Darmstadt, 24.01.2022	
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Abstract

A promising approach to evaluate the toxicity of wastewater is the use of species representing different trophic levels. The duckweed "Lemna minor" is one of the most commonly used aquatic plants in toxicity testing procedures for testing various inorganic, organic chemicals and their mixtures in aqueous solutions.

The already conducted research projects on the suitability of the Lemna minor test for paper mills effluent have reported a poor reproducibility of the test results with the same sample, even in the same laboratory. Moreover, untraceable elevated toxicity values of effluent samples from graphic paper mills with deinking processes resulted within these projects.

The aim of this thesis is to investigate the main parameters influencing the result of the Lemna minor test. Subsequently, the suitability of the "Lemna minor test" on the phytotoxic effect of paper mills effluent can be evaluated on this basis. For this purpose, the standardized Lemna minor test including sample preparation, conservation conditions and test procedure is defined. Followed by multiple screening in seven paper mills to investigate the influence of the production process, wastewater treatment technique, and wastewater parameters on Lemna minor test results. The repeatability of the test results is investigated by three times repeating the test under standardized conditions for clarified effluent of seven paper mills in the same laboratory. Moreover, the possibility to reduce the Lemna minor test duration is conducted within this study.

Based on obtained results within the standardization of Lemna minor test, the clarified effluent samples from paper mills investigated by Lemna minor test should either be analyzed directly or be frozen after sampling. It is possible to store frozen clarified effluent samples up to two weeks prior to testing. Following the obtained results, the execution of the Lemna minor test using four dilution levels is recommended (D1, D2, D4 and D8). Prior to testing, sample filtration must be conducted via a black ribbon paper filter, and their pH be adjusted to 5.5. As a result of the test, G_w values are calculated, which reflect the first nominal dilution level (highest concentration of effluent sample) at which growth inhibition does not exceed 10 %.

The reduction of growth inhibition along the wastewater treatment process could be observed for all investigated paper mills, which corresponds to the effectiveness

of current treatment in paper mills. Compared to the clarified effluent samples, the untreated and partially treated wastewater samples are both found to have higher COD, AOX, and turbidity values. In correlation with higher contaminations, these samples showed increased growth inhibitions of Lemna minor. In this study, it could not be determined whether one of these factors or an interaction of several factors accounted for the higher growth inhibition.

In the course of repeatability investigations, six of seven tested clarified effluent samples showed $1 \leq G_w \leq 2$ based on the frond area and G_w value of 1 based on frond number. There is still no limit value for toxicity tests in the wastewater ordinance. However, in most toxicity tests using fish embryos, algae, or Luminescence bacteria the value of 2 is considered a harmless wastewater sample [1]. Accordingly, the resulted G_w values within this work can prove the harmlessness of the investigated clarified effluent samples and the repeatability of the test results under standardized conditions.

Within the investigation of the reduction of the Lemna minor test duration, the same G_w values are obtained after five and seven days of the experiment. Due to the low toxic effect of investigated paper mills clarified effluent on Lemna minor almost the same growth inhibition can result after five days. However, this finding is only valid for clarified effluent samples of the investigated paper mills. The samples with higher toxicity can have different results.

Kurzfassung

Ein vielversprechender Ansatz zur Bewertung der Abwassertoxizität ist die Verwendung von Spezies, welche die verschiedenen trophischen Stufen repräsentieren. Die Wasserlinse "Lemna minor" ist eine der am häufigsten verwendeten Wasserpflanzen in Toxizitätstestverfahren zur Prüfung verschiedener anorganischer und organischer Chemikalien und deren Mischungen in wässrigen Lösungen.

Bei den bereits durchgeführten Forschungsprojekten zur Eignung des Lemna minor-Tests für Papierfabriksabwässer wurde eine schlechte Reproduzierbarkeit der Testergebnisse mit derselben Probe, sogar im selben Labor, festgestellt. Darüber hinaus wurden im Rahmen dieser Projekte bisher nicht erklärbare erhöhte Toxizitätswerte von Abwasserproben aus grafischen Papierfabriken mit Deinkingverfahren festgestellt.

Ziel dieser Arbeit ist die Identifikation von Einflussfaktoren auf das Ergebnis des Lemna minor-Tests. Auf dieser Grundlage findet anschließend eine Bewertung der Eignung des Lemna minor-Tests für die Bestimmung der phytotoxischen Wirkung von Papierfabriksabwässern statt. Zu diesem Zweck wird ein standardisierter Lemna minor-Test mit Probenvorbereitung, Konservierungsbedingungen und Testverfahren für die Endabläufe der Papierfabriken entwickelt. Anschließend wird ein Mehrfachscreening zur Bestimmung des Einflusses des Produktionsprozesses, der Abwasserbehandlungstechnik und der Abwasserparameter auf das Ergebnis des Lemna minor-Tests in sieben Papierfabriken durchgeführt. Der Lemna minor-Test wird unter den standardisierten Randbedingungen in demselben Labor dreimal wiederholt und die Wiederholbarkeit der Ergebnisse für die Endabläufe aus sieben verschiedenen Papierfabriken quantifiziert. Außerdem wird im Rahmen dieser Studie die Möglichkeit der Reduzierung der Lemna minor-Testdauer von sieben auf fünf Tage untersucht.

Nach Auswertung der gewonnenen Ergebnisse für die Standardisierung des Lemna minor-Tests sollen die Abwasserproben nach der Entnahme entweder direkt analysiert oder eingefroren werden. Die gefrorene Abwasserprobe kann bis zu zwei Wochen konserviert werden. Die Testdurchführung für die Endabläufe wird mit vier Verdünnungsstufen empfohlen (V1, V2, V4 und V8). Die Proben müssen vor dem Test schwarzbandfiltriert und der pH-Wert der Proben auf 5,5 eingestellt werden. Die Darstellung der Ergebnisse erfolgt als G_w-Wert, der die nominell

erste Verdünnungsstufe (höchste Konzentration an Abwasserprobe) beschreibt, bei der eine Wachstumshemmung von 10 % nicht überschritten wird.

Für alle untersuchten Papierfabriken konnte eine Reduzierung der Wachstumshemmung im Verlauf der Abwasserbehandlung beobachtet werden. Diese entspricht der Effektivität der aktuell verwendeten Abwasserreinigung bei den Papierfabriken. Im Vergleich zu den behandelten Abwasserproben ergeben sich für die unbehandelten und die teilweise behandelten Abwasserproben höhere Werte für den CSB, den AOX und die Trübung. Die höheren Belastungen dieser Abwässer sind mit höheren Wachstumshemmungen verbunden ($G_w \geq 12$). In dieser Studie konnte nicht abschließend geklärt werden, ob einer dieser Faktoren oder eine Interaktion mehrerer Faktoren für die erhöhte Wachstumshemmung verantwortlich ist.

Im Rahmen der Wiederholbarkeitsuntersuchungen zeigten sechs von sieben untersuchten Endabläufe $1 \leq G_w \leq 2$ bzgl. der Frondfläche und einen G_w-Wert von 1 bzgl. der Frondanzahl. Es gibt noch keinen Grenzwert für Toxizitätstests in der Abwasserverordnung. Bei den meisten Toxizitätstests mit Fischei, Algen oder Lumineszenzbakterien gilt jedoch ein Wert von 2 als unbedenkliche Abwasserprobe [1]. Dementsprechend können die im Rahmen dieser Arbeit ermittelten G_w-Werte die Unbedenklichkeit der untersuchten Endablaufproben und die Wiederholbarkeit der Testergebnisse unter standardisierten Bedingungen belegen.

Bei den Untersuchungen zur Reduzierung der Lemna minor-Testdauer von sieben auf fünf Tage für die Endabläufe aus sieben Papierfabriken werden aufgrund deren geringer toxischen Wirkung die gleichen G_w-Werte erzielt. Allerdings ist diese Erkenntnis nur für die untersuchten Endablaufproben gültig. Bei Proben mit erhöhter Toxizität können davon abweichende Ergebnisse resultieren.

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Table of Contents

1 Introduction

The pulp and paper industry has become one of the largest economic sectors in the world and is also one of the most water-related industries [2]. Paper consumption has increased steadily over the last few decades. The world production of paper in 2020 was about 401 million tons [3]. This industry releases a wide range of pollutants into the environment, making it the sixth-largest polluter after oil, cement, leather, textile and steel industries [4]. The water utilized in pulp and paper mills depends significantly on the characteristics of the raw material, type of produced paper and the extent of water reuse [5]. In Germany, the specific volume of paper mills wastewater has decreased significantly in recent years. While the average specific wastewater volume in 1970 was still around 50 l/kg paper, by 2020 it had dropped to less than 9 l/kg paper [6].

The reduction in the specific volume of wastewater in recent years mainly corresponds to the development of the paper industry and its water cycle [7]. However, it is not always possible to maintain the product quality at the same level by simultaneously minimizing the water cycle [8].

The completely closed water loops have barely been used in the paper industry. According to a survey in 2016, only 4 % of the paper products in Germany are manufactured in facilities with a closed water circuit. This segment has so far mainly included corrugated board manufacturers [8, 9].

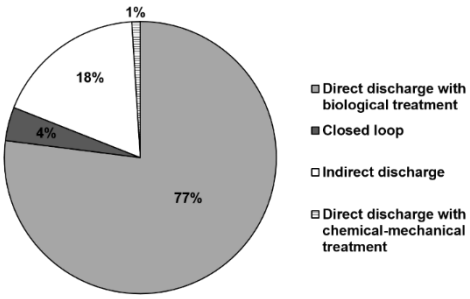


Figure 1: Percentage breakdown of wastewater treatment in the German paper industry, based on the product volume produced in 2016 [7].

Although the specific wastewater volume of the paper industry has been significantly reduced in recent years, wastewater treatment in the paper industry is still of great importance. Due to the large volumes to be treated, it is still obvious why efficient wastewater treatment is a prerequisite in the paper industry.

The wastewater quality in the paper industry is controlled by various EU-wide and national regulations to limit their potential risk to the environment. Annex 28 of the Wastewater Ordinance "Manufacturing of Paper and Board" is also framed by these regulations [10]. In addition to the provided chemical and physical analyses in Annex 28, biological tests are becoming increasingly important for assessing the toxicity of wastewater. It should be noted that 22 of the 53 annexes in the AbwV already contain specifications regarding biotests for the regulated industry [1]. For the paper industry, this is not yet the case, although discharge notices often include a requirement for a fish embryo toxicity test according to DIN EN ISO 15088:2009 [11].

The major advantage of biotests compared to the pure consideration of wastewater parameters is that biotests allow a complete consideration of the wastewater constituents and their toxicological effects [12]. In particular, both attenuating and enhancing effects of the water constituents on each other can be recorded [12].

After the restriction of the use of animals for toxicity tests in 2006, many studies were conducted to investigate the toxicity effect of wastewater on plant organisms [13]. The use of plant species as primary producers for toxicity tests plays an essential role in early warning systems. In this way, contaminations can be intercepted in advance, allowing rapid intervention before biomagnification processes occur along food chains or the contaminants has a chance to spread [13]. Among different plant species, the duckweeds “Lemna minor” are frequently used as a toxicity test organism for numerous inorganic and organic chemicals and their mixtures in aqueous solutions. Lemna minor can be found all over the world in different temperature zones. In addition, its physical properties such as small size, high reproduction rates and vegetative propagation make it an excellent test system [14]. The Lemna minor test has been proposed as an assessment criterion for paper mill effluents based on an evaluation of the project sponsored by the German

Federal Environment Agency (UBA) between 2006 and 2008 [15, 16]. The inclusion of the Lemna minor test was strongly discussed during the revision of Annex 28 of the Wastewater Ordinance (AbwV), but was ultimately not implemented.

The conducted projects on the investigation of Lemna minor test as a toxicity assessment for paper mills effluents reported poorly reproducible results for the same effluent sample even in the same laboratory. Moreover, within these studies, incomprehensible elevated toxicity values for effluent samples of graphic paper mills with the deinking process were recorded [1, 17]. For this reason, the suitability of the Lemna minor test as a toxicity assessment for paper mills effluent could not be explained yet.

The aim of this work is to investigate the suitability of the Lemna minor test for determining the phytotoxic effects of paper mills effluent. For this purpose, the Lemna minor test under different test conditions was performed. Subsequently, the test conditions that allow reproducible test results were defined as a standard method of toxicity testing of clarified effluent of paper mills.

First part of this work focuses on a literature review on critical chemical parameters contained in paper mills wastewater as well as wastewater treatment methods commonly used in the paper industry and their efficiency to remove critical parameters listed in Annex 28.

This is followed by an investigation of paper mills effluent by the Lemna minor test at the PMV institute. For this objective, all major parameters influencing the result of the Lemna minor test are identified and quantified. These parameters include the sample preparation method, the influence of preservation time and temperature as well as test procedure on the growth rate of Lemna minor. After the analysis of the parameters affecting the result of the Lemna minor test, the standardized test procedure is outlined.

The third part of this work describes the differences in the test results with respect to the dilution levels (G_w) for various paper mills as well as for different wastewater treatment techniques. In addition, wastewater parameters (Chemical oxygen demand COD, total suspended solids TSS, Adsorbable organic halides AOX etc.) are measured and their correlation with growth inhibition of Lemna minor is discussed.

Special focus will be placed on the repeatability of the results, as this has been a problem in previous investigations. This is realized by repeating the test three times under standardized conditions for seven different paper mills.

The final part of this work is addressing the concern if whether it is feasible to reduce the Lemna minor test duration to five days. This issue is investigated and discussed by comparing the test results after five and seven days for clarified effluent samples of seven different paper mills.

2 Theoretical backgrounds and state of the art

2.1 Review the paper mill operation

The main raw material for paper production is the fiber made of wood or paper for recycling, depending on the type of produced paper and the used production technology. Other materials such as straw, hemp, grass and other cellulose-bearing material can be used [18]. The process starts with stock preparation, which converts raw stock into finished stock for the paper machine. In this step, different fibers are mixed together and fillers, sizing agents, dyes and other additives are added and intensively mixed with the paper stock [19]. The desired solid content or stock consistency is adjusted by dilution of paper stock [20]. The stock is then fed to the paper machine, if necessary further additives, e.g. retention agents can be added. The Fourdrinier paper machines are still used widely in the paper industry. However, for many paper grades, they have been replaced with twin-wire machines (e.g. gap formers), which allows the machine to operate at a much higher speed [19]. The main components of a paper machine are headbox, forming section, press section, dryer section, calender stack and reel-up [21]. The papermaking process starts at the headbox, where the paper stock is injected into the forming wire. In the Fourdrinier process, the sheet is formed by distributing the paper stock on one horizontal wire from the headbox. While in twin-wire machine, the paper stock is directed between two wires operating at the same speed [19]. The fiber suspension in the headbox consists of about 99 % water and only 1 % fibers and fillers. In the forming section, the water is removed from the fiber suspension and the paper sheet is formed. Following the forming and dewatering sections, the sheet has a relative dryness of 15 – 25 %. The water that runs off is collected and returned to the production process [19–21]. The process is then followed by a press section, where further pressure is applied to the wet paper web to remove additional water and consolidate the sheet into an approximately 40 % consistency. In the press section, the specific volume, opacity and smoothness of the paper can be controlled [20]. Following the press stage, the paper goes through a lengthy, steam-heated drying stage. Here, water is removed through evaporation, and fiber-fiber bonds are formed as the paper is rolled around large-diameter, hot cylinders. In the final section of the paper machine, a series of roll-nips reduce the thickness and smooth the surface of the paper-sheet before it is wound up on the

reel [20, 22]. **Figure 2** describes the whole process in the Fourdrinier paper machine.

Further paper finishing processes including surface coloring, coating and impregnating can be carried out directly in the paper machine or after reeling in separate machines [20, 23].

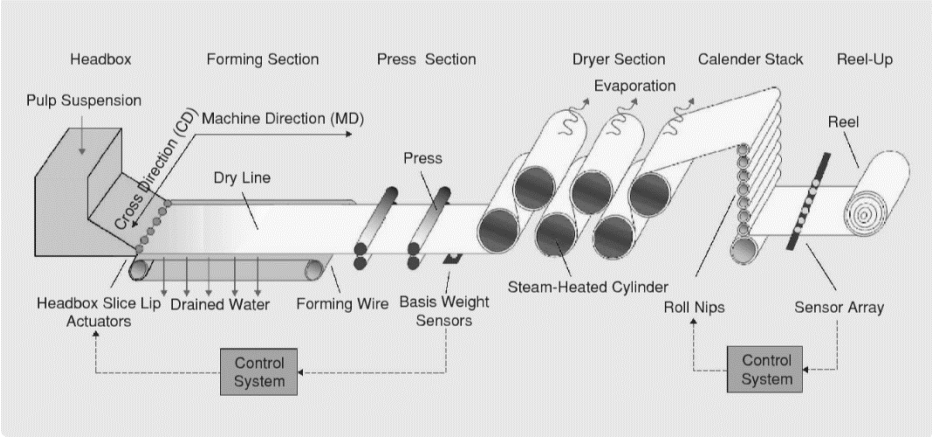


Figure 2: Schematic of a Fourdrinier-type paper machine with the most important stages and selected control loops [22].

2.2 Water and wastewater in paper mill operation

Large quantities of water are required in paper production. In papermaking, water is used as a suspending agent, transport medium for fibers and fillers and solvent for chemical additives [8]. Depending on the paper type, between 250 and 1000 liters of water per kg of product are required to prepare the fibers and form the paper web in the paper machine. However, most of this water can be reused [8]. **Figure 3** illustrates the example of a water loop system for paper production. The water collected from different processes of the paper machine (see **2.1**) is directed to save all filter, where its contained fibers and filler are separated and returned to the stock preparation. The most common treatments applied in save all filter for internal water reuse are filtration and dissolved air flotation. Based on the quality

of the treated water in this step, it can be used as recirculation water for stock preparation and paper machine or discharged as residual wastewater [24].

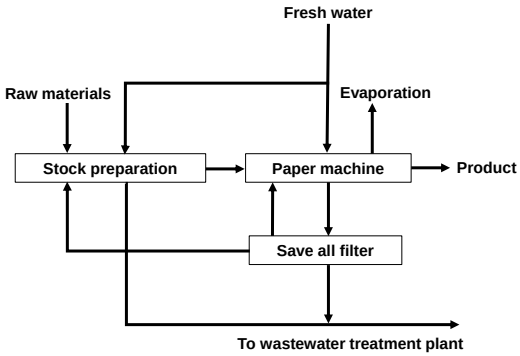


Figure 3: Schematic representation of water loop in paper mills. Stock preparation is the process of making paper stock from raw materials, fillers, additives, etc. and adjusting the stock consistency by dilution with water. Save all filter is the process of recycling the used materials in stock preparation and treatment of the process water for internal use.

Fresh water is required in small quantities at various stages in the production process. Depending on the type and quality of the produced paper, an additional amount of fresh water is used to adjust the purity of the circulation water required for production. Additionally, the evaporation losses must be compensated by fresh water [8, 24].

Wastewater is produced during paper production as excess recirculated water, which is displaced by fresh water. The resulting wastewater contains a load of water-soluble substances. However, the concentration of dissolved organic and inorganic substances in wastewater depend significantly on the produced paper type [25, 26].

The wastewater from the paper mills can contain an assortment of additives, including mineral filler products, like calcium carbonate, clays and titanium dioxide. During the production of corrugated board and in paper mills using recycled paper as raw material, substantial amounts of starch can enter the wastewater treatment plant, which is one of the main reasons for the high organic load in the wastewater [27, 28]. Furthermore, the wastewater resulting from paper mills contains sizing

agents, like resin products, alkenyl succinic anhydride, or, alkyl ketene dimer, which are typically supplemental to fiber suspension in form of emulsions, and not all of this is preserved in the paper product. In the case of colored papers, dyes are of high importance in terms of wastewater treatment [29].

2.3 Wastewater treatment in paper industry

Biological wastewater treatment using conventional aerobic and combined anaerobic-aerobic wastewater treatment processes is the typical form of treatment for paper mills wastewater [30]. The main structure of wastewater treatment in the paper industry can be seen in **Figure 4**.

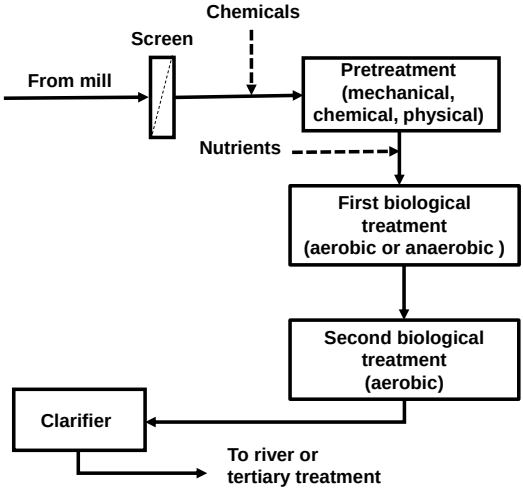


Figure 4: Schematic of the general wastewater treatment plant in paper mills. Depending on the used wastewater treatment system in paper mills, the addition of chemicals in pretreatment and addition of nutrients for biological treatment can be implemented.

At the beginning of the clarification process, the untreated wastewater from production enters a mechanical or chemical pretreatment. Initially, solids such as excess fibers or filler residues are removed by means of different techniques such as

sedimentation, filtration, etc. This can be followed by chemical precipitation, neutralization stage and cooling of the wastewater to reduce the pH and temperature to the level required for biological treatment. Since the paper mill effluents contain a low amount of nitrogen (N) and phosphorus (P), nutrients must be added to the wastewater before it is directed to the biological treatment process. The nutrients are added in form of urea, phosphoric acid, ammonium salts, phosphates and diammonium hydrogen phosphate to the wastewater [8, 30, 31].

The next step is biological treatment. Biological treatment in paper mills consists in particular of two stages. The first stage can be either an aerobic or an anaerobic process, followed by the second biological treatment in the form of aerobic treatment. Consequently, the partially treated wastewater is directed to a clarifier where it is finally purified by a sedimentation process. If necessary, the third treatment stage is performed, using techniques such as biofiltration, ozone stage or chemically assisted flotation. The main goal of the third treatment stage is to further improve the quality of wastewater beyond the limitations of conventional technologies. This can include the removal of nutrients such as phosphorus and nitrogen, reducing the high percentage of suspended solids and other matters, which could not be fully treated within a biological treatment [8, 31].

The most common methods and aggregates for wastewater treatment in paper mills and their effects on the quality of wastewater are discussed in the following chapters.

2.3.1 Anaerobic wastewater treatment

The anaerobic wastewater treatment describes a process of biodegradation in which no air or oxygen is supplied, this is analogous to the fermentation process [32–34]. In chemical terms, the process of anaerobic digestion for a typical wastewater treatment operation can be divided into the steps of hydrolysis, acid formation, acetogenesis with the production of hydrogen and carbon dioxide, and methanogenesis [35, 36]. A different group of microorganisms is involved in each degradation step, and they partly depend on each other to deliver substrates and consume degradation products, respectively. Microorganisms performing hydroly-

sis and acidogenesis are responsible for the first step. During the fermentation process, they produce volatile fatty acids, acetate, hydrogen, and carbon dioxide. A crucial and rate-limiting step in this process is hydrolysis, especially for complex materials such as lignocellulosic material and biological sludge. The majority of fermentation products are converted into acetate, CO_2 and hydrogen. These are utilized by methanogens to form biogas (CO_2 and CH_4) in the final step [37].

The application of the anaerobic process is recommended for a COD concentration of higher than 2 g/l [8]. If this value is not available in the overall wastewater stream, but a sub-wastewater stream with a higher COD concentration exists, its separate treatment before discharge into the aerobic biological treatment stage can reduce not only the load but also the tendency to form bulking sludge [8].

Similarly, the inlet temperature of the anaerobic reactors must be between 35 °C and 38 °C to guarantee an optimal biocenosis for the process. The pH value in anaerobic reactors is usually adjusted between 6.8 and 7.2 to ensure that the influent is not preloaded with organic acids [8].

Total suspended solids (TSS) are considered problematic in the anaerobic process based on their accumulation in the reactor [8]. This leads to the displacement of biomass from the system and thus to a reduction in reactor performance. The concentration of TSS should usually be less than 50 mg/l. Another crucial aspect of anaerobic treatment is the high calcium concentrations in wastewater [8]. These can react with CO_2 produced by the microorganisms to form calcium carbonate and precipitate at an elevated pH value compared to the inlet in the reactor [8]. Particularly, a decrease in biological activity is observed in reactors without biomass carriers. Therefore, the calcium load for the anaerobic process should be as low as possible. Furthermore, the sulfate concentration must be kept low, since it causes toxicity to the methane-producing bacteria [38]. For the anaerobic process, the following condition regarding sulfate concentration in inlet flow should apply: Sulfate concentration/COD concentration = 0.1 [8].

In addition to the above mentioned conditions for the anaerobic process, reactors are also differentiated according to their structures and efficiencies as well as the use of carrier materials on which the microorganisms are grown, whereby methods without carrier materials predominate in the paper industry [8].

As can be seen from **Figure 5** for anaerobic reactors, more than half of the pulp

and paper industry uses the currently widely available technologies of IC (internal circulation) and EGSB (expanded granular sludge bed) reactors. The remaining market is shared by UASB (upflow anaerobic sludge blanket) reactors and contact process reactors such as CSTR (continuous stirred tank reactor) [37].

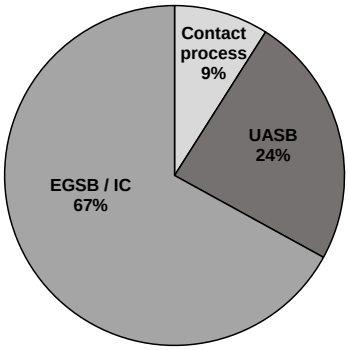


Figure 5: Percentage distribution of reactor types used for anaerobic wastewater treatment in the pulp and paper industry, n = 417, stand 2017 [36].

The remarkable advantages of anaerobic treatment are the elimination of the costs for aeration of the system and the energy production in the form of methane [35]. Moreover, the amounts of produced sludge are generally less than in conventional aerated biological treatment systems. Furthermore, it has lower chemical consumption and smaller land requirements because of its smaller reactors size [30, 36]. The amount of produced sludge in anaerobic treatment is 0.04 kg Biomass/kg removed CSB, this value is about 15 times higher in an aerobic process [8]. Anaerobic processes produce biogases, whose main components are methane (65-80 vol. %) and CO_2 (20-35 vol. %) [9, 31]. Even so, recovering and reusing biogases, such as methane and hydrogen, as a source of fuel during full-scale treatment can provide considerable economic benefits to the treatment plants [39]. Anaerobic wastewater treatment can effectively reduce the COD content up to 80 % [9]. **Figure 6** describes the percentage conversion of COD in anaerobic compared to aerobic wastewater treatment.

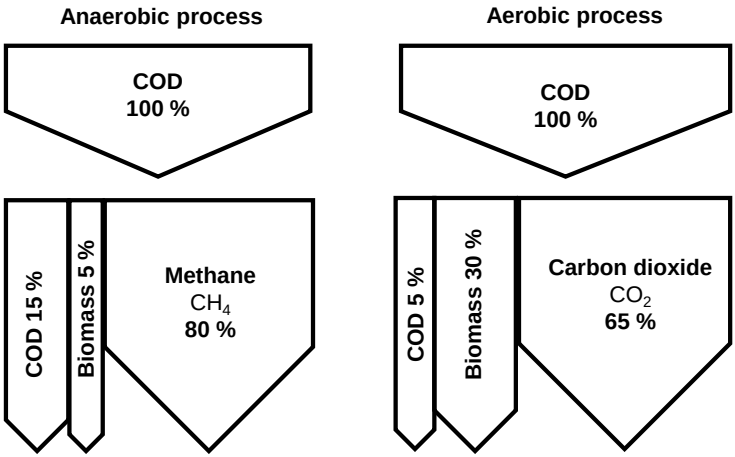


Figure 6: Percentage conversion of COD in anaerobic and aerobic wastewater treatment [8].

The main limitation of many anaerobic treatment processes is that they are not sufficient enough to remove some contaminants, such as nutrients or disease-causing microorganisms (pathogens) within the wastewater. All anaerobic processes provide only partial treatment and therefore the effluent needs to be treated further before releasing into the environment. Furthermore, the investment costs of this process are 20 to 30 % higher compared to aerobic treatment [9].

A crucial concern associated with anaerobic processes is the possibility of reducing sulfate ions to other compounds like sulfides or H_2S [40]. These compounds are produced through the action of sulfate-reducing bacteria [41, 42]. Methane production can be interfered by reduced sulfur compounds [43]. Among sulfide species, H_2S is considered the most toxic to methanogenic communities responsible for the production of methane. Moreover, concrete corrosion in full-scale reactors is another problem associated with sulfate-reducing bacteria [44]. Because sulfur compounds can inhibit anaerobic biological treatment, it is recommended to strip H_2S gas from the system and purge it [43].

Another disadvantage of anaerobic treatment is that the reduction of AOX throughout this process indicates the slow degradation of toxic compounds and chlorinated compounds are still present in the effluent of anaerobic treatment [29].

In almost all pulp and paper industries, the anaerobic wastewater treatment process is followed by aerobic treatment [45]. Many researchers have evaluated the performance of an anaerobic-aerobic treatment process superior or at least identical to aerobic treatment and as an appropriate way for the treatment of paper mills wastewater [46–48]. The removal of residual organic matter from anaerobically treated wastewater can be successfully achieved by aerobic treatment. This sequential combination can also reduce the total production of sludge and produce biogas that could be further used for energy production in the mill. In other cases, the combination of these treatments with membrane filtration can be used to further polish the effluent for possible reuse as a replacement for fresh water in the mill [46].

2.3.2 Aerobic wastewater treatment

The aerobic treatment stage is used in the paper industry either in combination with anaerobic treatment or as a solely biological treatment stage.

The basic principle of aerobic treatment is to use oxygen (O_2) for converting organic carbon compounds in wastewater into carbon dioxide (CO_2), water (H_2O) and biomass [31, 49]. This process utilizes microorganisms (mainly bacteria) that are selectively enriched in the aggregates to convert the organic carbon compounds [50]. Because of their high oxygen consumption, the supply of atmospheric oxygen and also pure oxygen is required [51]. The limiting factor for the growth of microorganisms is the availability of carbon, oxygen, phosphorus, nitrogen and nutrients in wastewater [8]. The addition of nutrients takes place at the beginning of the biological treatment. The ratio of BSB_5 : N : P in the aerobic stage should be in the range of 100 : 5 : 1 to 100 : 3 : 0, 5 [31, 52]. These values are controlled by parameter measurements at the effluent of the biological wastewater treatment, as well as by analyses of the biomass and the activated sludge [52]. In addition, the temperature should be in the range of 25 °C to 38 °C, and the pH value should be between 6.5 and 8.5 [8].

As in anaerobic wastewater treatment, excess sludge is formed when various microorganisms degrade organic substances [49]. The value of approx. 0.6 kg bio-

mass/kg of degraded COD can be expected for the aerobic process [31]. The degradation of 90 % to 98 % of BOD_5 and 80 % to 95 % for COD can be achieved by means of aerobic treatment plants (see **Figure 6**) [9].

The high quota of carbohydrates in the paper mills wastewater causes a strong tendency for the formation of bulking sludge. Bulking sludge is characterized by a high level of filamentous bacteria, which cause poor settling and thus high sludge volume. Consequently, the separation in the clarifier will be poor and a large fraction of biomass will be lost with the effluent. This problem is overcome by the suspended carrier process, as it involves a biomass carrier, which allows high concentrations of biomass to be processed. By retaining the carrier material in the reactor through a sieve in the outlet, only the excess suspended biomass is leaving the reactor with the (partially) biodegraded wastewater [9].

Another major point in aerobic wastewater treatment is the removal of the nitrogen compounds such as ammonium [51]. However, this is necessary to prevent toxic effects on aquatic life and avoid oxygen depletion (in water bodies) during nitrification [51]. Furthermore, oxygen consumption should take place in a controlled environment (e.g. in an aeration tank) [51]. In this process, ammonium is first converted to nitrate via nitrite, and then in the denitrification step, it breaks down into nitrogen molecular, which removes the nitrogen from the wastewater. For complete denitrification, 1.7 kg oxygen/kg ammonium is required [52].

To address the issues mentioned above, some researchers have suggested a second stage of aerobic treatment. This can be named “staged” aerobic treatment [53, 54]. It is imperative that the system is designed in such a way that active biological organisms can settle on suitable support surfaces in the second stage of treatment. During the second stage of treatment, predator species (protozoa and metazoa, including rotifers) can proliferate and feed on the bacterial matter, which arose in the absence of predation during the first stage of treatment. It is stated that in the second stage, both suspended solids and sludge solids were substantially reduced [53].

The last survey on the different reactor types used for aerobic treatment in the paper industry in Germany was conducted in 2006. According to this survey, the vast majority of the paper industry uses activated sludge tanks, followed by aeration cascade, biofilter, and moving bed reactor. Only 11 % of paper mills use the other processes for aerobic wastewater treatment (see **Figure 7**).

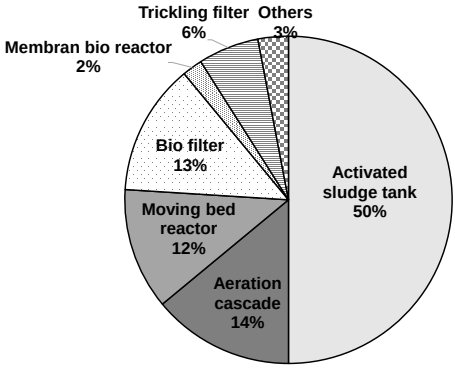


Figure 7: Percentage distribution of reactor types used for aerobic wastewater treatment in the paper industry, stand 2006 [54].

2.3.3 Advanced wastewater treatment

Advanced treatment is a step to finish up a wastewater treatment process, which mainly focuses on discharging the wastewater with parameters that can meet the environmental regulations. The tertiary or advanced treatment of wastewater is used to remove specific constituents of the wastewater that cannot be removed by primary or secondary treatment. This type of treatment is sometimes referred to as tertiary treatment because it usually comes after highly effective secondary treatment. However, in some cases advanced treatment processes are combined with primary or secondary treatment processes (e.g., by adding chemical compounds to primary clarifiers or aeration basins to remove phosphorus) [29, 55].

Different treatment processes can be used to remove nitrogen, phosphorus, additional suspended solids, and refractory organics or dissolved solids from wastewater. Advanced wastewater treatment in the pulp and paper industry is mainly performed by ozone treatment and membrane filtration techniques such as micro-, ultra- or nanofiltration and reverse osmosis [30, 55, 56].

2.4 Characterization of paper mills effluent

The paper industry discharges a large amount of wastewater into the environment. Therefore, characterization of the composition of the discharged effluent and its potential risk to aquatic and land ecosystems is of high importance. An exact qualitative and quantitative determination of individual constituents of wastewater is practically impossible due to the variety of contained substances. Therefore, the sum parameters such as COD, TOC, AOX, TN_b, etc. are established for analysis and evaluation of wastewater. In addition, the use of biotests for complete consideration of the wastewater constituents and their effects on each other is reported by many researchers [57, 58]. Since predominantly wood and recycling papers are used as fibrous material for paper production, the paper mills effluent contain a high load of organic substances [30]. The chemical oxygen demand (COD) is used as a sum parameter for the analysis of organic water constituents [59]. The used raw material, chemical additives and internal water purification and recirculation systems can be mentioned as influencing variables on the COD value of the paper mills wastewater [2, 60]. Usual values for clarified effluent are in the range between 80 and 500 mg/l COD depending on the type of produced paper [8].

The dyes and used chemical additives, as well as the naturally halogen compounds contained in the wood are the main source of adsorbable organically bound halogens (AOX) in the wastewater [61]. The specific AOX load in papers containing wood ranges from 0.9 to 5.4 g/t and in papers containing recycling paper from 0.9 to 7.5 g/t [8]. Nitrogen and phosphorus contents in paper effluents are mainly derived from nutrients used for biological treatment of the wastewater. However, this can be reduced in most cases by modifying the nutrient dosage. In addition, additives can cause the increase in the concentration of nitrogen and phosphorus. A specific phosphorous load of 0.01 to 0.04 kg/t and a specific load of nitrogen from 0.01 to 0.8 kg/t can be expected in the clarified effluent of paper mills [8].

The complex mixtures of organic and inorganic compounds have been detected in the paper mills effluent in recent studies. These compounds contribute not only to toxicity and increase the COD value, but some of them have endocrine-disrupting properties (EDCs) [58, 62, 63]. Some of these hormone-like substances, which can cause various adverse effects in humans and the environment, are used in small quantities for paper production and processing or occur as impurities in other chemicals [64]. In addition to the natural lignans and phenolic compounds derived

from wood, these substances include representatives of phthalates, bisphenols, and polycyclic aromatic hydrocarbons (PAHs) [64]. Within the research projects, INFOR Project No. 70 and AiF Project No. 15181 N the existence of endocrine-disrupting substances in biologically treated wastewater from paper mills could be proven [64–66]. In the Water Framework Directive (WFD), endocrine disruptors are classified as priority substances in Annex X [67]. In the Community Strategy for Endocrine Disruptors (COM (1999) 706 final), the European Union has already determined the goal of creating regulatory measures for protection against endocrine disruptors and is currently setting standards worldwide with its legal framework conditions [68].

One of the most important sources of contaminations in paper mills wastewater are the chemicals used as additives to improve the performance of the material or the production process. The addition of chemicals may vary depending on the production technology and the sort of produced paper. Furthermore, using recycling paper as an important raw material for paper production can cause different pollutions in process and wastewater [69]. The quantified chemicals in paper mills effluent included phthalates, phenols, polychlorinated biphenyls, and different toxic metals (Cd, Co, Cr, Cu, Ni, and Pb) [69]. The current results demonstrate large variations in the concentration of chemicals depending on the recycling paper fraction and produced paper. However, a lack of quantitative data on the presence of chemicals in produced wastewater from paper mills is evident in the literature.

Wood pulping may slightly elevate the concentration of heavy metals in wastewater due to the presence of metals that occur in higher concentrations in wood (e.g., iron, manganese, copper) [70]. The concentration of metals naturally occurring in wood can be different depending on the plant species, the part of the plant used in paper making, and local soil conditions [64, 71]. Furthermore, inks, pigments, coating, or impurities can be a source of metals in paper mills wastewater [72]. The concentration of heavy metals in paper mills effluent is typically negligible. However, some heavy metals like copper and cadmium, even in micro concentration, can negatively influence aquatic life and plants [73].

Aluminum sulfate is used as an additive in the paper industry. In paper mill effluents, sulfate concentrations of up to 600 mg/l can generally be expected. For paper mills with a wood free program, this value can be as low as < 300 mg/l. In the case

of performing classical resin sizing, which is now hardly used, the sulfate concentration lies between 300 and 600 mg/l. However, the sulfate concentration strongly depends on the specific wastewater volume [8].

For paper mills with low specific wastewater volumes, the sulfate concentrations close to the above mentioned upper limit (600 mg/l), and also higher up to > 1,000 mg/l are observed. Another source of sulfate are recovered papers used for papermaking. An evident example of this are mills producing corrugated board, where aluminum sulfate is typically not used because of its adverse effect on certain quality parameters, but still sulfate concentrations between 600 and 1,000 mg/l can be found in their wastewater [8].

2.5 Current status of the regulation of chemicals in paper mills effluent

The quality of wastewaters produced in the paper industry is controlled by various EU-wide and national regulations to limit their potential risk to the environment. Furthermore, these regulations serve as the framework for Annex 28 of the Wastewater Ordinance on the manufacture of paper and board [10]. In the current valid version of Annex 28 of the Wastewater Ordinance (AbwV) for paper and board production, requirements are given for the following parameters:

- Total suspended solids (TSS)
- Biochemical oxygen demand in 5 days (BOD_5)
- Total nitrogen (N_{tot}) as the sum of ammonium, nitrite and nitrate
- Total nitrogen bound (TN_b)
- Total phosphorus (P_{tot})
- Chemical oxygen demand (COD)
- Total organic carbon (TOC)
- Adsorbable organic halogen compounds (AOX)

Furthermore, in all cases, the wastewater temperature "T_{effl}" and the pH value are limited by the local approval authorities. In addition to the above mentioned parameters, biological tests for assessing the toxicity of wastewater are becoming in-

creasingly important. This is not yet considered a requirement for paper mills effluent. However, some paper mills use this test to ensure the quality of the treated wastewater before discharging it to environment.

The inclusion of biological test in Annex 28 was discussed many times during the revision process but was not concluded in the final version. It remains to be seen to what extent the biological test will play a role in a later revision of Annex 28.

The advantage and disadvantages of different biological tests and their application in paper industry are discussed in detail in section **2.6**.

2.6 The use of biotests to assess aqueous sample toxicity

The biological test method (biotest) is an approach for detecting environmental hazards and assessing the aquatoxicological effects of aqueous samples (e.g. wastewater sample) at different trophic levels [29]. Particularly, the biotest is utilized by using plant species or different organisms, which are exposed during their growth phase to different concentrations of the toxicants for a few days [14, 74–76]. The combined use of conventional chemical analyses and biological monitoring techniques allows a comprehensive assessment of the water quality [13].

In traditional toxicological studies, the emphasis was placed on evaluating the toxic potential of contaminants existing in water, especially concerning human health. From this, the use of acute fish test (AFT) with various kinds of test organisms (e.g., rainbow trout, zebrafish, etc.) because of their similarity to the human organ has been used widely as a toxicity assessment for water and wastewater in the last decades [77]. According to REACH (EC, 2006), the use of animals for toxicity tests has been hardly restricted and the urge for alternatives has been intensively discussed in recent years. One of the alternatives for using adult or juvenile fish mentioned in OECD test guideline 203 is to study acute toxicity effects in fish embryos [78]. For this purpose, two validation studies on fish embryo toxicity tests (FET) were conducted on behalf of the German Federal Environmental Agency. These studies reported a good correlation of median lethal concentration (LC50) values of FET with the AFT and conducted that both AFT and FET have a similar sensitivity to tested chemicals. Consequently, OECD published the test guideline 236 for the

determination of FET in July 2013 [78]. Based on that, the most used toxicity test in the paper industry in recent years is the fish embryo test. Accordingly, the result of this test is often issued in discharge notices of paper mills. Here, the requirement is $G_{Ei} = 2$ in most cases. However, some studies in recent years reported that fish embryos are less sensitive to certain chemicals (particularly to neurotoxic substances) than adult or juvenile fish [79–81]. According to these studies, for acute toxicity of neurotoxins, respiratory syndrome is an important factor that is lacking in embryonic fish. Apparently, fish embryos are provided with oxygen independent of their cardiovascular system [82]. Consequently, in contrast to adult fish, FETs are unlikely to exhibit a reduction in oxygen supply due to neurotoxic substances. Also, this can be crucial for the paper industry, as some biocides used in this industry are carcinogenic, neurotoxic, and harm reproduction and cell development, especially in the early stages of life [83].

The use of plant-based tests is discussed and considered as applicable water quality monitoring in environmental toxicology in the last two decades [84–87]. The remarkable advantage of using the plant for toxicity tests is that they can respond to toxic substances earlier than other organisms. This plays an essential role in early warning systems to capture the contamination in advance and before the process of biomagnification along the food chain [13]. More than 6,000 aquatic species are tested to be utilized in water ecotoxicology assessment. Among them, microalgae by 60 % are the most used species in water assessment, followed by flowering plants at 33 %, macroalgae about 6 %, pteridophytes ≈ 1.6 %, and aquatic bryophytes with 1 % [13]. **Table 1** summarizes the most used plant species for investigation of different substance groups dissolved in an aqueous sample.

Choosing an appropriate biotest to evaluate the toxic effects of the water or wastewater sample being tested, it is crucial to consider the capability of the species to tolerate the presence of certain contaminants as well as bioaccumulate them in their tissues [13]. Numerous freshwater plant organisms possess these properties, and they can also be exploited very effectively in the ecotoxicology sector when determining the level of contamination in the sample. The above-mentioned advantages of using plant organisms for toxicity tests make them an ideal alternative for FET and the subject of many studies in recent years. Among different plant organisms used for toxicity tests, particular focus was placed on duckweeds (Lemna minor) because they can be easily cultured in different temperature zones and are sensitive to a wide range of toxicants. The function and advantages of the

Lemna minor test are described in detail in **2.7**. In addition, some studies are executed to investigate the suitability of the Lemna minor test for monitoring the phytotoxic effects of paper mills effluent [88]. These will be discussed in chapter **2.8**.

Table 1: The list of most used test species for toxicity testing of dissolved substance groups in aqueous samples. The toxicity test for each test species is evaluated based on its observation parameters [13].

Investigated substances	Test species	Observation parameters
Heavy metals	Myriophyllum spicatum	Growth rate (phytomass production, length/frond)
	Lemna minor	Growth rate, frond number, dry weight
	Lemna gibba	Enzymatic activity, chlorophyll fluorescence
Pharmaceuticals	Lemna spp.	Enzymatic activity, pigment analyses, chlorophyll fluorescence
	Lemna minor	Frond number, frond area, fresh weight
	Myriophyllum sibiricum	Root length, wet weight, dry weight, root number, longest root, node number, plant length, pigment analyses
Hydrocarbons	Desmodesmus spp.	Photosynthetic activity, enzymatic activity
	Lemna gibba	Inhibition of photosynthetic activity
Pesticides	Eichhornia crassipes	Fresh matter, leaf anatomy
	Chlamydomonas reinhardtii	Growth rate, dry weight, photosynthetic pigments, protein contents, enzymatic activity
Surfactants	Lemna minor	Growth rate, frond area, frond number, chlorophyll fluorescence
	Chlorella vulgaris	Cell density, chlorophyll content
Plastics	Lemna minor	Growth rate, chlorophyll content, root length, root cell viability
	Scenedesmus	Growth rate, photosynthetic efficiency

2.7 Lemna minor and its sensitivity to water constituents

The duckweed Lemna minor is one of the most commonly used aquatic plants in toxicity testing procedures for testing various inorganic and organic chemicals and their mixtures in aqueous solutions [89]. In its ecological function, duckweed is the primary producer at the beginning of the food chain for waterfowl, fish and small animals [16]. Lemna minor is a small vascular plant that grows rapidly, easy to culture, and is sensitive to a wide variety of toxicants, which makes it ideally suited for carrying out the toxicity test procedure. Furthermore, Lemna minor can be found all over the world. It grows in stagnant waters in the tropics through the temperate zone to the Arctic. The leaflets up to 3 mm long have air-filled hollow bodies so that able them to float on the water surface. On the backside, the plants have a root thread up to 15 mm long that protrudes into the water and is used to absorb minerals and nutrients from the water. Duckweeds Lemna minor grows in so-called colonies, which consists of a mother frond and one to three daughter fronds. In this context, the frond describes the individual leaf analogue structure of a duckweed colony, which is capable of reproduction [16, 90].

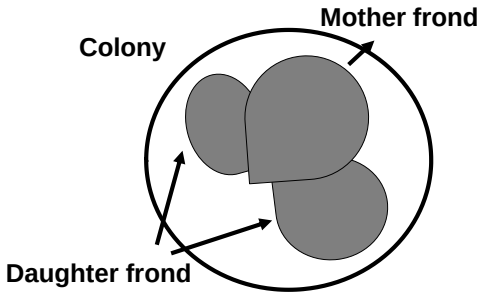
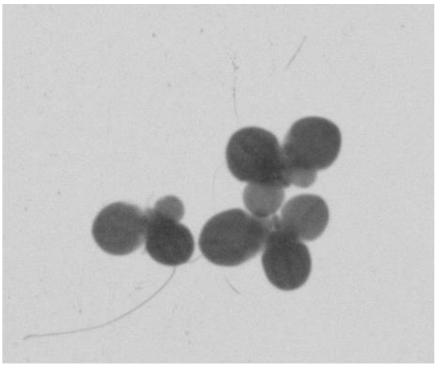
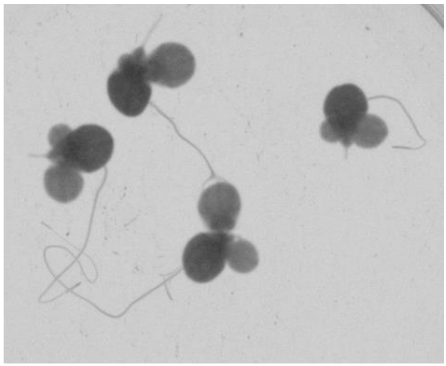


Figure 8: Schematic of a Lemna minor colony [91].



Number of colonies: 3
Frond number: 10
Frond area: 67.92 mm²



Number of colonies: 4
Frond number: 11
Frond area: 75.45 mm²

Figure 9: Schematic of Lemna minor under camera. The pictures were taken in two different times and before starting Lemna minor test.

Due to the REACH regulation on priority substances according to the EU Water Framework Directive (Directive 2000/60/EC) and the Environmental Quality Standards Directive (Directive 2008/105/EC), numerous studies based on biotests have been published in the last decades [88]. In the following, the effect of water and wastewater contaminants on Lemna minor is discussed.

Lemna minor can absorb and accumulate heavy metals. In some papers, this is described as a potential and cost-effective method for heavy metal detoxification [92, 93]. According to studies, heavy metals, especially copper and cadmium, even at very low concentrations (≥ 0.4 mg/l) contained in water or wastewater can influence the growth rate of Lemna minor [73].

The ability of Lemna minor to selectively denitrify is also the topic of many investigations [91, 94]. However, NH_4^+ concentrations of 20 mg/l in interaction with pH values above 8.0 and thus the increasing influence of free NH_3 inhibit the growth of Lemna minor. Although biomass growth still occurs at low pH values of 5 - 6, the leaves show first signs of chlorosis (yellow color, loss of pigment) or even necrosis (death of fronds). Paper mills use many chemicals to control bacteria and fungi,

such as chlorinated hydrocarbon pesticides (DDT, dieldrin, endrin), polychlorinated biphenyls (PCBs), and biocides containing phenol and benzene derivatives, which sometimes severely inhibit duckweed growth to the point of complete plant death [13, 57, 95].

It is known from studies on endocrine disruption using in-vitro test with modified yeast cells [65, 66] that biologically treated wastewater from paper mills, albeit at other trophic levels, can certainly have negative (mostly oestrogenic) effects on test organisms. The native effect of these substances on Lemna minor is reported in some studies, but not for paper mills effluent [96, 97]. To what extent these substances, partly natural phenolic and other wood constituents, and also industrial chemicals, which may be present in trace concentrations in wastewater from waste paper processing, can also have an effect on Lemna minor growth is not known.

The effect of micro- or nano-plastics on Lemna minor is also investigated in some research [13]. These reported phytotoxicological effects of the most commonly encountered plastics include photosynthesis inhibition and sprout and root growth of the plant. However, many of these studies demonstrated that plant species are generally only affected when micro and nanoplastics concentrations are higher than those recorded in nature [98, 99].

The influence of substances in an aqueous sample on the growth of Lemna minor is described by percentage inhibition thresholds below which no toxicity effect is observed to the test organism. The results of the Lemna minor test are given as G_w value, which corresponds to the dilution level at which a 10 % inhibition of Lemna minor growth is not exceeded compared to the control.

2.8 Investigation of paper mills effluent with Lemna minor test

As described in previous sections (see **2.6** and **2.7**), Lemna minor has remarkable advantages that make it an ideal test organism for toxicity testing of water and wastewater samples. The use of the Lemna minor test for the evaluation of toxic effects of various chemicals along the interaction of substances in an aqueous solution received a lot of attention in recent years, and it is used widely for toxicity assessment [13]. However, few studies focus on the suitability of the Lemna minor test for paper mills effluents.

The results of the investigations of Lemna minor in the wastewater of paper mills conducted by UBA from 2001 to 2007 showed G_w = 1 or 2, but also maxima up to 8 [17]. However, these investigations indicated high sensitivity and low cross-sensitivities of Lemna minor compared to other bioassays investigated (luminescent bacteria, fish embryo, daphnia, algae, and Umu or Ames test) [17]. Based on this and on the recommendation of UBA, the inclusion of the Lemna minor test was intensely discussed in the process of the revision of Annex 28 of the AbwV. For this purpose, some studies are performed to investigate the applicability of the Lemna minor test for paper mills effluent.

The first study was carried out by Christian Schuessler (2012/13) [100]. The focus of the work was on the introduction of a test procedure to facilitate the use of the test in future studies [100]. Various problems were also encountered in this investigation. The validity criteria listed in DIN EN ISO 20079:2006 [16] could not be fulfilled during this work. In addition, an increase in G_w values was observed, while no clear trend of the values over the dilution levels was recognizable. Based on the obtained results in this work, the effluents from paper mills with deinking demonstrated higher G_w values.

The investigation of the toxicological effect of paper mills effluent on Lemna minor was carried out by Magdalena Wandinger in 2013 [101]. In this study, the results of the Lemna minor test were compared with the luminescent bacteria test as an acute bioassay. There is no inhibition reported by using luminescent bacteria test, whereas the Lemna minor test resulted in high inhibition of plants. In this case, Wandinger suggested to check the investigations with another bioassay designed for chronic toxicity [101]. Moreover, a comparison between effluents of paper mills with and without deinking plant was performed in this work. As a result, higher toxicity for paper mills with deinking plant was recorded. However, due to a high results variation, the increased toxicity could not be clearly attributed to the processes of the deinking plants [24]. Within the scope of this study various problems due to frequent contamination of the test samples by algal growth (Chlorella vulgaris) occurred. Further results concerned the formation of biofilms on the surfaces of the test samples, the appearance of which resulted in strong growth inhibition. Reproducibility also proved to be problematic in her investigations, as different G_w values were sometimes determined in investigations of the same sample in different laboratories.

In the period from 2012 to 2014, two subsequent investigations on growth inhibition of Lemna minor growth inhibition test in relation to paper mills effluent were carried out at PTS by Oeller [1]. The aim of the first study was to determine the effect of untreated, partially and also full biologically treated wastewater from the paper industry on the test organism Lemna minor. Furthermore, the substances leading to the increases in the G_w values had to be identified. Another objective was to define a new limit value (G_w) that could be included in the revision of the AbwV. In the course of the study, 49 samples from 15 different mills (two of them fresh fiber mills) were analyzed. The G_w values could be narrowed down to the following ranges through the investigations:

- Untreated wastewater: $G_w \leq 2…24$
- Anaerobically treated wastewater: $G_w \leq 2...6$
- Fully treated wastewater: $G_w \leq 2...8$

Among investigated fully treated wastewater samples, the higher G_w values were observed for samples treated with the anaerobic-aerobic process. Increases in G_w values to 12 or 24 occurred only in wastewater samples of graphic paper production. However, a clear assignment of the increase in G_w values was not possible due to the limited database. Based on the investigations, Oeller once again pointed out the weak concentration-effect relationship in the Lemna minor test. Furthermore, he described the problematic reproducibility in repeat tests or comparative studies with external laboratories. Therefore, further investigations are still required. Due to the uncertain reproducibility and the identified need for further research, Oeller also advised against the inclusion of the Lemna minor test in the revision of the AbwV. The originally proposed limit value of $G_w = 8$ was accordingly rejected after this work.

According to studies, Lemna minor test is described as a reliable toxicity test with reproducible results. Therefore, the main research question after conducting research in the paper industry was to find the parameters, which can potentially cause the deviation of test results.

For this purpose, further investigations were conducted by PTS to study the effect of different chemical additives used in the paper industry on the Lemna minor test [17]. In this research project a total of 22 different chemical additives from all relevant substance groups were investigated for their phytotoxic effect on the Lemna

minor test. Nine of these selected additives were subjected to aerobic biological treatment and tested again on Lemna minor. All 22 additives tested in form of commercial substances demonstrated a large variation in G_w values. In contrast, low G_w values were recorded for defoamers and one of the felt cleaners. Biocides and retention aids/colorants showed high G_w values. The G_w values of the four poorly degradable additives tested in the project (fixing aid, biocide, process/wastewater treatment aid, strength enhancing additive) remained almost constant or decreased by just one dilution level through biological treatment. Since each product group contains a wide variety of products, none of the additive types proved especially critical or insignificant for Lemna minor. An essential aspect for the final evaluation of chemical additives can be the interaction of the factors such as dosing quantity, retention and biodegradability, which could not be investigated in this work. Accordingly, a clear assignment of increased Lemna minor toxicities to specific additives or additive groups could not be realized. However, the author reported that chemical additives used in papermaking are rather unlikely to lead to an increase in G_w values in biologically treated paper mills wastewater.

Based on conducted results from studies, the critical question regarding the parameters causing the variation in Lemna minor test results for paper mills effluent could not be answered, yet.

3 Problem description and strategy of this work

This chapter discusses the relevance of this work, its main objectives and the strategy to achieve them.

As discussed in previous chapters, the paper industry is the sixth major polluter in the world and discharges a huge amount of water into the environment. Even with the most efficient and modern operational techniques, the production of one ton of paper requires 60 m^3 of water, resulting in at least 50 m^3 of wastewater [4]. Although current wastewater treatment can remove most of the critical substances contained in wastewater, a suitable bioassay to study the negative environmental impact of these substances is of high importance.

The studies already conducted on the suitability of the Lemna minor test as a biotest for paper mills effluent are associated with partially poor reproducible results for the same effluent sample even at the same laboratory. The test procedure of previous investigations were all based on DIN EN ISO 20079:2006, however, the following points were not noticed and clarified in detail by them:

- The effluent samples were mainly taken by paper mills personnel and were sent in frozen form to the laboratory. However, the exact time between sample receipt by the laboratory and the start of the test as well as the conservation conditions (time, temperature) at this time was not clarified.
- According to DIN EN ISO 20079:2006, adjusting the pH value for effluent samples with pH between 5.0 and 8.0 is not required. However, in some investigations, the pH value was adjusted to 5.5. So far, there is no information about the necessity or influence of pH value on the results of Lemna minor tests.
- There is no method reported for separating solid and colloidal substances from wastewater in DIN EN ISO 20079:2006. While in previous studies sedimentation of samples for a determined time has been considered in some laboratories.
- Due to high variation in the obtained results of previous investigations, the required number of dilution levels for each test series is not determined yet.

The first experimental part of this work focuses on the investigation of the influence of sample conservation, sample preparation as well as the test procedure on the Lemna minor test result. Within the scope of this investigation, the influence of

pH value, conservation temperature, conservation time, test preparation and required number of dilution levels are determined and discussed. The selection of investigated paper mills clarified effluent for this part is based on previous research. As a result, the clarified effluent sample treated with the anaerobic-aerobic wastewater treatment technique was reported as the most toxic sample, followed by the clarified effluent sample from mills with the deinking process.

The second experimental part of this work focuses on the description of the toxicity of wastewater samples along the wastewater treatment stages. This part can additionally describe the difference between wastewater quality resulting from paper mills with different products as well as the efficiency of wastewater techniques (anaerobic-aerobic and aerobic-aerobic) for removing and treating critical substances. Furthermore, the measured chemical and physical parameters of samples from different treatment stages are used to define the correlation between these parameters and the resulted growth inhibition of Lemna minor.

In the next part, the repeatability of the Lemna minor test results for clarified effluent of paper mills in the same laboratory is investigated. This investigation used the standardized test method described in the first part of the work. Under defined conditions, the Lemna minor test is repeated three times for clarified effluent samples of seven different paper mills. The repeatability is then be demonstrated by the obtained G_w value after each repetition for the investigated paper mills. Moreover, the variation of the results within the three times investigations is indicated in a box whisker plot.

The last part of this study describes the influence of time on the obtained test results. The aim is to investigate, whether a reduction of the test duration from seven to five days can result in a different growth inhibition percentage as well as G_w value. This is performed by analyzing the clarified effluent samples after five and seven days from seven different paper mills.

4 Equipment, materials and methods

4.1 Equipment

The following equipment is used for the Lemna minor test procedure and evaluation of the test results.

Test procedure:

- **Plant growth cabinet** (KBW 720, Co. Binder): According to DIN EN ISO 20079:2006, the plant growth cabinet is required for Lemna minor test to guarantee the constant temperature and light conditions for plants during the test.
- **Beakers** with a minimum volume of 200 ml (2/3 of the total volume is used for test solutions).
- **Glassware** is used for preparing various dilution levels of the wastewater sample and nutrient medium (volumetric flasks, graduated cylinders, graduated pipettes, Petri dishes).
- **Uniform glass covers** are used to avoid accidental contamination and to minimize evaporation losses of test solutions in beakers during the test.
- **Photometer** (Pharo 300, Co. Spectroquant®).
- **pH-value measuring device** (3210, Co. WTW).
- **Conductivity measuring device** (3110, Co. WTW).
- **Autoclave** (CertoClav Laboratory autoclave Classic).
- **Black ribbon filter paper** (MN 640 w, Co. Machery-Nagel), filter material: cellulose, retention capacity: 7 – 12 µm, ash content < 0.01.
- **Membrane filter paper** (RC 55, Co. Whatman), filter material: regenerated cellulose, pore size: 0.4 µm.
- **Centrifuge** (8K10, CO. Sigma).

Test evaluation:

- Light table with Basler industrial camera and image analysis software MedeaLAB Count & Classify version 6.7, Co. Medea AV Multimedia und Software GmbH, Erlangen.
- MATLAB software version R2018b.

4.2 Lemna minor culture

The Lemna minor stock culture was provided by the German Federal Environment Agency (UBA, Berlin) in a solid culture medium.

The used Lemna minor for the toxicity test was grown five to seven days before the start of the test in Steinberg medium and stored in the plant growth cabinet. The depth of the medium should be at least 3 cm. In order to minimize the likelihood of a lag phase due to interactions between the plants, it should be ensured that less than 50 % of the total available surface area is covered with plants.

During the Lemna minor test preparation phase with untreated and partially treated wastewater in the institute laboratory (PMV), massive algae contamination occurred first in the cultivation basin and then in the test samples. Microscopic images showed that the green algae is absorbing on the roots of the Lemna minor, cutting them off from nutrient uptake and causing the Lemna minor to die. By consistently implementing the countermeasures described below, it was possible to prevent further algal contamination during this study:

- Spatial separation of plant cultivation and test performance.
- Heating the plant growth cabinet at 70 °C for at least 4 hours after each test (every 7 days) and disinfecting it with isopropanol.
- Change Steinberg medium in the cultivation tank twice a week.
- Separation of all glassware used for Lemna minor test from the other used glassware in the laboratory. These have to be washed in a laboratory dishwasher after each test and be heated to 120 °C.

The Lemna minor pre-culture used for the toxicity tests had to fulfill the following criteria:

- Exponential growth,
- The 7-fold increase in the frond number of Lemna minor pre-cultures have to be proven in seven days (i.e. $r \geq 0.275$ per day or doubling time ≤ 2.5 days),
- The pre-culture must consist of young, fast-growing colonies with a strong green color, without any visible damage, chlorosis or necrosis.

4.3 The modified Steinberg nutrient medium

According to DIN EN ISO 20079:2006, the modified Steinberg medium was used as a medium for storage and test. This medium is composed of eight stock solutions and distilled water. The composition of the stock solutions is described in **Table 2** and **Table 3**.

Table 2: Stock solutions (macro elements) used for Steinberg medium.

Macro elements	Concentration (g/l)
Stock solution 1:	
KNO_3	17.50
KH_2PO_4	4.5
K_2HPO_4	0.63
Stock solution 2:	
$MgSO_4 \cdot 7H_2O$	5.0
Stock solution 3:	
$Ca(NO_3)_2 \cdot 4H_2O$	14.75

Table 3: Stock solutions (micro elements) used for Steinberg medium.

Micro elements	Concentration (mg/l)
Stock solution 4:	
H_3BO_3	120.0
Stock solution 5:	
$ZnSO_4 \cdot 7H_2O$	180.0
Stock solution 6:	
$Na_2MoO_4 \cdot 2H_2O$	44.0
Stock solution 7:	
$MnCl_2 \cdot 4H_2O$	180.0
Stock solution 8:	
$FeCl_3 \cdot 6H_2O$	760.00
EDTA Dinatrium-dihydrate	1,500.00

The stock solutions were then autoclaved and stored in the refrigerator to be used for the Lemna minor pre-culture and test procedure.

The 1 l Steinberg medium used for cultivation and storage of Lemna minor was prepared with 20 ml of stock solutions 1 to 3 and 1 ml of stock solutions 4 to 8, which were added to approx. 900 ml of distilled water in a 1 l volumetric flask.

The 10-fold concentrated Steinberg medium was used for the experiment. With respect to 100 ml, 20 ml each of stock solutions 1 to 3 and 1 ml of stock solutions 4 to 8 were added to approx. 30 ml of distilled water in a 100 ml volumetric flask.

4.4 Analysis of the wastewater sample for Lemna minor test

In order to quantify the influence of the chemical and physical wastewater parameters on the test results, these parameters were measured before the start of each test. **Table 4** summarizes these parameters and the applied analytical methods. The samples used for cuvette tests were filtered via black ribbon filter paper before analysis.

Table 4: Measured wastewater parameters including the particular analytical method used.

Parameter	**Analytical method**
COD	Cuvette test Merck-DIN ISO15705
P_{tot}	Cuvette test Merck HC901316
NO_2-N	Cuvette test Merck HC015947
NO_3-N	Cuvette test Merck HC913062
NH_4-N	Cuvette test Merck HC014924
TSS	DIN 38409 T. 2
TN_b	DIN 38409 T.27
AOX	DIN EN ISO 9562
Turbidity	DIN EN ISO 7027-1:2016
Surface tension	Drop shape Analyzer (DSA100, Co. Kruess)

4.5 DIN standard for the application of the Lemna minor test

The execution of the Lemna minor test was based on DIN EN ISO 20079:2006. However, the number of tested samples and the measuring days were varied from the DIN standard in order to quantify more samples. No evidence of abnormalities associated with this modification was noted during this study.

5 Experimental procedure

5.1 Establishment of the Lemna minor test

The Lemna minor test was carried out according to the DIN EN ISO 20079:2006. For this purpose, the temperature in the plant growth cabinet was set to 24.0 °C with spatial temperature deviation of maximum ± 1 °C. To ensure the uniform illumination of all test approaches, the spatial distribution of photosynthetic active radiation was measured by means of a spherical ball sensor on several days. The measured mean value of 100 $\frac{\mu E}{m^2 \cdot s}$ at the height of liquid surface in beakers was within the recommended value of the DIN standard of 85 $\frac{\mu E}{m^2 \cdot s}$ - 135 $\frac{\mu E}{m^2 \cdot s}$ with a deviation of 5 %.

The plant growth cabinet consists of three levels. The beakers are positioned in special storage boxes with the capacity of 32 beakers per box, so that there are 96 available places in the cabinet. The black color and closed construction of the boxes minimizes the incidence of side and reflected light.

Figure 10: Schematic of the plant growth cabinet incl. the black storage boxes.

At the beginning of each Lemna minor test series, different dilution levels were prepared with a final volume of 500 ml in volumetric flask. The 450 ml of each dilution level was divided into three beakers to ensure three parallel determinations of 150 ml of sample with exactly the same mixing ratio. 3.5-dichlorophenol with the concentration of 3.06 mg/l was used as reference sample (according to the concentration specifications of DIN EN ISO 20079:2006). In addition, control samples consisting of 10-fold concentrated Steinberg medium and distilled water were used for each test series. Subsequently, the pH value of the control, reference and dilution samples were adjusted to 5.5 ± 0.2. For this investigation, six different dilution levels were tested within each test series. The preparation of the test samples is shown in **Figure 11**.

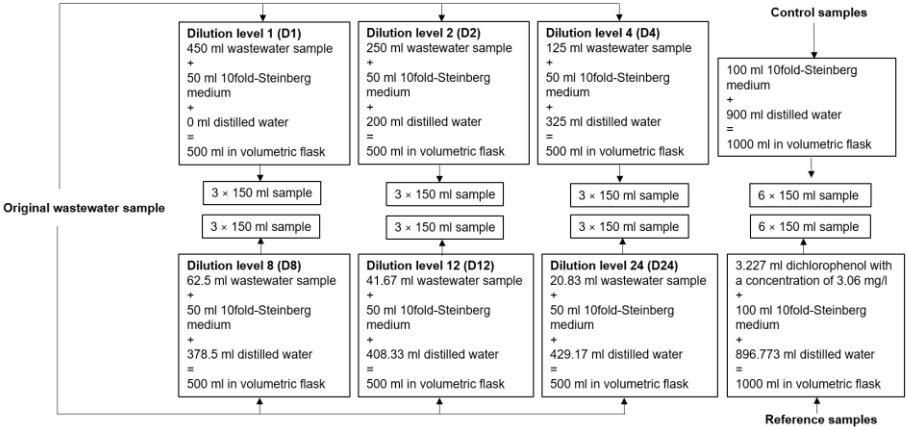


Figure 11: Sample preparation for Lemna minor test.

In the next step, three to four healthy and green-colored Lemna minors with a total of ten to sixteen fronds were added to each beaker filled with the previously prepared samples. The beakers were then covered with glass covers and kept in the plant growth cabinet for seven days.

In order to test the repeatability of the test results, the Lemna minor test was carried out in three different times under same conditions by using the same sample. The most significant point for the investigation of repeatability of the test results is to test the same sample under defined conditions at different times. The actual wastewater sample can deviate in its chemical and physical parameters with each

sampling time. On the other side, conservation of the wastewater sample can also result in the change of properties and deviation from the test results. Thus, the test was performed by using a filtrate of the disintegrated paper sample instead of a wastewater sample. This sample can provide comparable properties as the paper mill effluent and the same parameters for each test repetition. Furthermore, this provides a sample with the same properties for each test repetition. In addition to the paper sample, control and reference samples were tested in each experiment series. For this purpose, paper sample was defibrated according to the PTS method RH-014/2015 ref [102]. First, the corrugated board sample (testliner) was torn into small pieces under standard conditions; 24 g of it weighed and soaked in 1 l of distilled water for 2 h at room temperature. Subsequently, 1 l of distilled water was added, and the mixture was whipped in the disintegrator according to ISO 5263. The whipped pulp was then filtered via black ribbon filter paper and diluted to have a COD value between 100 mg/l and 200 mg/l. The sample preparation for Lemna minor test is identical to **Figure 11**.

5.2 Monitoring the growth rate of Lemna minor

The Lemna minor test duration according to DIN EN ISO 20079:2006 is seven days. To determine the growth rate of Lemna minors, the number of fronds and frond area were determined. These were measured at the beginning and end of the test and additionally after two and five days. For a better comparison, measurements were done at approximately the same time on each measurement day.

The measurement of the Lemna minor growth rate was carried out by using the image analysis system (Count & Classify MedeaLab, Co. Medea AV Multimedia und Software GmbH) at the beginning of this work. The system was not sufficiently stable, and the measurements were partially inaccurate. Therefore, the device was only used to take pictures of the Lemna minors in each test sample. The analysis as well as the measurement of frond number and frond area were executed with a developed algorithm using MATLAB software at the PMV institute [103]. The measurement of the frond number is done by manually selecting the individual frond. The program counts the marked objects and the total frond number in each test sample and exports the result in an excel file. To determine the frond area, the images are imported into the software, and the original RGB images are converted into the HSV colour space. Each frond is characterized by the software as a cylindrical object. In the HSV color space, cylinder sectors are defined that have maximum and minimum values for hue H, saturation S, and value V. The cylinder sectors tightly constrain the color spectrum of Lemna minor. The used values for this work were H between 54 ° and 108 °, S between 25 % and 100 %, and V between 0 % and 100 %. It should be noted that these values are set as a default based on the experimental setup and lighting conditions and are not universal. Based on the HSV values for each pixel, the algorithm determines the pixels as Lemna minor or background. Subsequently, the software sums up all Lemna minor pixels and converts them via a calibration factor into the frond area in mm^2. The measured values for the frond area of the loaded pictures can then be exported in an excel file.

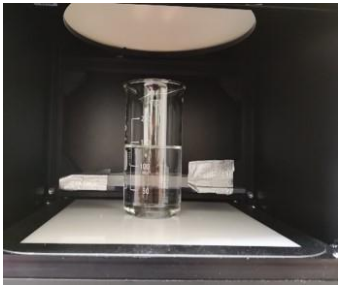


Figure 12: Adjustment of the sample under camera.

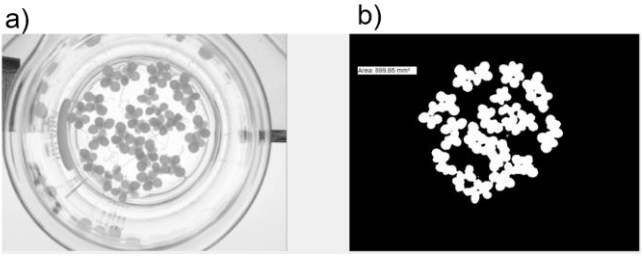


Figure 13: **a)** Schematic of Lemna minor under camera **b)** Measurement of frond area by MATLAB software.

5.3 Evaluation of the test results

The aim of the experiment is to characterize the growth rate of Lemna minor. For this purpose, two observation parameters - frond number and frond area - are compared on different days with one of the previous daily measurements. The increase in frond number and frond area is described by the growth rate r in equation (1) [16]. However, this equation is only valid when the exponential growth rate of Lemna minor in control samples can be proven within seven days of the experiment.

$$r = \frac{\ln(x_{t2}) - \ln(x_{t1})}{t_2 - t_1} \quad (1)$$

where:

r: Growth rate per day
x: Value of the observation parameter: frond number or frond area
x_{t1}: Value of the observation parameter after t_1 days
x_{t2}: Value of the observation parameter after t_2 days
$t_2 - t_1$: Period between x_{t2} and x_{t1} in days

The percentage inhibition of the growth rate for each test concentration is calculated according to equation (2) [16]:

$$i_r = \frac{r_c - r_t}{r_c} \times 100 \quad (2)$$

Where:

i_r : Inhibition of the mean specific growth rate in percent (%)
r_c : Mean specific growth rate of the control samples
r_t: Mean specific growth rate of the test concentrations

According to DIN EN ISO 20079:2006, a growth inhibition of less than 10 % is considered as a harmless wastewater sample. An inhibition greater than 50 % corresponds to the death of the plants. The results for the wastewater sample are then given as a G_w value. The G_w value describes the nominally first dilution level (highest concentration on wastewater sample) at which a growth inhibition of 10 % is not exceeded.

5.4 Standardization of the Lemna minor test for paper mills effluent

5.4.1 Sample preparation and conservation conditions

According to DIN EN IS0 20079:2006, the pH adjustment for investigated samples with pH values between 5.0 and 8.0 is not necessary. However, the pH value of control and reference samples has to be adjusted to 5.5. In conducted studies for investigation of the suitability of Lemna minor test for paper mills effluent, the pH value for all samples including control and reference samples was adjusted to 5.5 [1, 100]. Within the studies, the pH value is not constant throughout the seven-day test period, however, its change can be decelerated by adjusting the pH value. It remains still not clear if the pH value can influence the growth rate of Lemna minor.

Previous studies investigating paper mills effluents with the Lemna minor test were performed by using frozen effluent samples, which were defrosted at room temperature and homogenized before starting the test. However, in some laboratories the tested effluent sample was sedimented for a specific time before the test. So far, nothing is known about the influence of the sample preparation on the test results. Due to the DIN EN ISO 20079:2006, the analysis of water constituents dissolved in the wastewater sample is essential for Lemna minor test. However, the influence of these parameters on the Lemna minor has not been clarified yet.

Within the scope of this work the influence of pH value, sample preparation, as well as wastewater conservation before the test, was investigated by testing wastewater samples from three different paper mills. The selection of the investigated paper mills was based on the results of previous studies. Therefore, paper mills that indicated a high G_w value were used for the standardization of the Lemna minor test in this study. Questionnaires were prepared and the general conditions of production and wastewater treatment technology were discussed in a personal interview at each examined paper mill. With the questionnaire, among other topics, the following information was recorded and considered (see **Appendix II**):

- Measured chemical and physical wastewater parameters on the sampling day as well as the monthly average of each parameter.
- The used raw materials (incl. chemical additives) for each produced paper type.

– The used wastewater treatment technology as well as flocculants, defoamers and other additives for plant configuration and operational management of the wastewater treatment plant.

Wastewater sampling was performed at all mills at least once personally. The responsible employees in the paper mills were then specifically instructed to be able to take and send the wastewater samples themselves for subsequent sampling times. The investigated clarified effluents in this work were mixed samples (2 h mixing). The samples were taken directly from the outlet of the clarifier in plastic bottles every 30 minutes. Subsequently, all bottles were mixed and filled into 2 l plastic bottles to be delivered to the laboratory. The paper mills were selected according to the following criteria:

– Investigation of the paper mills with different paper types production,
– Investigation of paper mills with different wastewater treatment techniques,
– Investigation of paper mills with and without deinking plants.

Table 5 summarizes the required information for a better understanding of the investigated paper mills. To ensure confidentiality, no further details on paper types, production volume, wastewater treatment plants, etc. are given. Nevertheless, in order to get an idea of the mill size, the investigated mills are divided into the following size classes according to production capacity.

– "Small" corresponds to ≤ 100,000 t per year.
– "Medium" corresponds to > 100,000 ≤ 250,000 t per year.
– “Large" corresponds to > 250,000 t per year.

Table 5: Investigated paper mills for standardization of the Lemna minor test.

Source	Paper mill code	Paper type	Paper mill size	Wastewater treatment
Recovered paper without deinking	A	Corrugated board	Large	Anaerobic-aerobic
Recovered paper with deinking	E	Graphic paper	Large	Aerobic-aerobic
	H	Recycled cardboard	Large	Aerobic-aerobic

The influence of pH value on the growth rate of Lemna minor was investigated for a pH range between 5.0 to 8.0 (investigated pH values: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0). It was crucial to use the same sample for all pH values investigation. Nevertheless, due to the capacity of the plant growth cabinet, examination of the sample with different adjusted pH values at the same time was not possible. The effluent sample can be different in its chemical and physical properties at various sampling times. Furthermore, storage of the sample can cause changes in effluent properties. Hence, for this investigation, the actual effluent sample could not be used. Thus, the test was performed using a filtrate of the disintegrated paper sample (sample preparation in **5.1**). This sample can provide comparable properties as the paper mill effluent and the same parameters for each test repetition.

The pH value for each sample was adjusted by using hydrochloric acid and sodium hydroxide before starting the test. After evaluating the results for tests with different adjusted pH values, two values, which could result in a higher growth rate, were selected. The influence of the selected pH values on the Lemna minor test over time was then investigated accordingly by using clarified effluent sample from paper mill A. Paper mill A was selected, as the high G_w values obtained by treating the wastewater sample with an anaerobic-aerobic treatment technique were evaluated in previous research. The effluent sample for this investigation was sedimented for 1 h after sampling and stored from one day to four weeks under frozen conditions. The reason for using sedimentation as a preparation method in this part was to separate only the coarse particles contained in the sample. Furthermore, in previous studies and for cases of turbid samples, 1 h sedimentation of the

samples was considered. Therefore, a better comparison of the obtained results in this work with previous studies could be achieved. Before starting the test, samples were defrosted at room temperature (min. 8 h is required) and the pH value was adjusted by using hydrochloric acid and sodium hydroxide.

The influence of the conservation temperature on the test result was characterized by storing the wastewater samples at different temperatures (freezer -18 °C, refrigerator 4 °C, room temperature 24 °C) for time durations ranging from one day to four weeks after sampling. For this investigation, clarified effluent samples were taken personally from paper mill A and conserved after 1 hour of sedimentation at different temperatures.

Since freezing the wastewater sample leads to an increase in total suspended solids (TSS), the effect of TSS value on the growth inhibition of Lemna minor as well as the change in the TSS value after one week of storage at frozen conditions was first investigated. For this purpose, the TSS value of the samples was measured directly after sampling (according to DIN 38409 T. 2), and the Lemna minor test was also executed on the same day without any treatment of the sample. A defined quantity of the same sample was frozen after sampling and stored in the freezer for one week. After one week, the TSS value of these samples was remeasured. Subsequently, the samples were homogenized in a 1 l beaker using a magnetic stirrer and used for Lemna minor test.

In the next step, various methods for separating the solid and colloidal dissolved substances were investigated. For investigation of the influence of the sample preparation method on the result of the Lemna minor test, the following methods were tested:

- **Sedimentation**: the wastewater sample was sedimented in a 1 l vessel for 1hour and then decanted.
- **Centrifugation**: the centrifuge containers (six pieces) were filled with approx. 400 ml of wastewater sample and centrifuged for 15 minutes at a speed of 5000 min^{-1}.
- **Filtration of the sample**: filtration of the samples was carried out with black ribbon filter papers which are suitable for coarse contaminants or particles (fast filtration), and with membrane filter papers which are used for very fine particles (see chapter **4.1**). The wastewater samples were filtered once by black ribbon filter paper and once by membrane filter paper. Filtration via

black ribbon filter paper was carried out with 1 l of wastewater sample. Whereas for membrane filtration, 500 ml of wastewater was used.

Figure 14 describes the investigated steps for sample preparation and conservation conditions of the Lemna minor test.

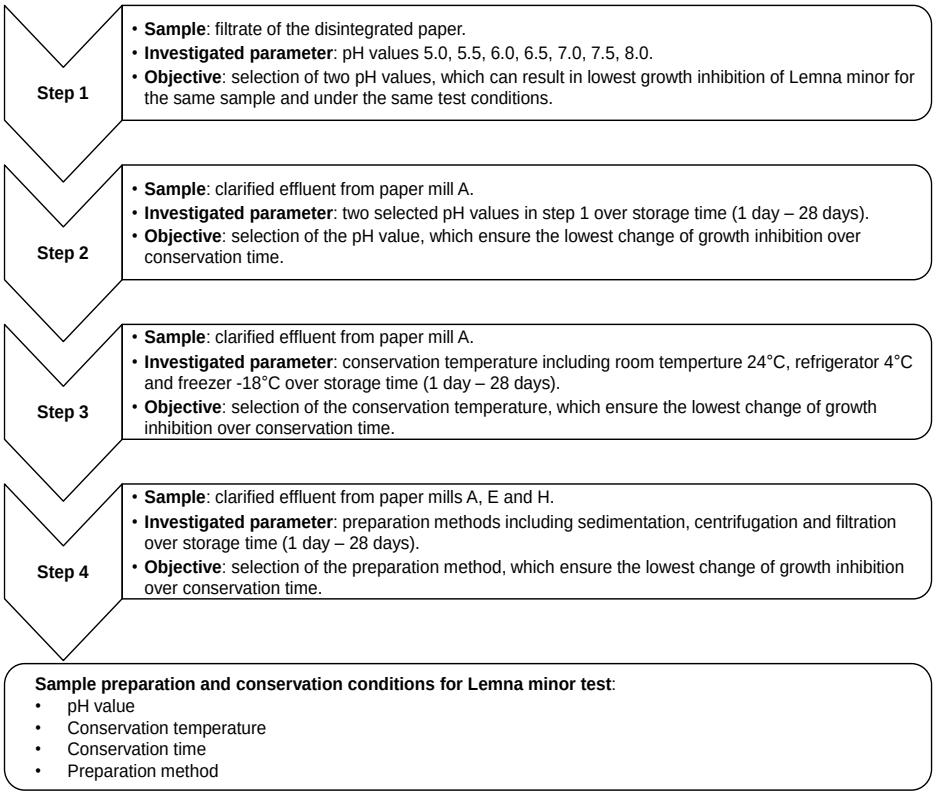


Figure 14: Investigated steps for definition of suitable sample preparation and conservation conditions of Lemna minor test.

5.4.2 The required number of dilution levels for the Lemna minor test

For each investigated effluent sample, six dilution levels (D1, D2, D4, D8, D12 and D24) were tested. To establish a reasonable test concentration range, the NOEC (No Observed Effect Concentration) was calculated for the tested effluent samples. The NOEC corresponds to the highest concentration at which no adverse effect on Lemna minor can be observed. If the objective is to calculate the NOEC, the lowest test concentration should be chosen, so that the growth of Lemna minor at this dilution level is not significantly lower than those growing in the control samples.

The ANOVA (Analysis of Variance) followed by Dunnett's test was applied to calculate the NOEC. The ANOVA calculates the mean specific growth rate and the residual standard deviation over the replicates for each test concentration. The resulting mean for each test concentration is then compared to the control mean using Dunnett's test as a suitable multiple comparison method (see **Appendix I**) [104].

5.5 Investigation of paper mills effluent using Lemna minor test

This chapter focuses on the investigation of paper mills by Lemna minor test. The objective of this study is to compare the wastewater samples from paper mills producing a comparable paper product at different treatment stages. Within this investigation, the effect of different paper type productions, as well as wastewater treatment techniques on the water quality can be evaluated. Furthermore, the change of G_w value along the wastewater treatment process for each paper mill can be observed. For this purpose, wastewater samples from seven paper mills were analyzed at four different treatment stages as demonstrated in **Figure 15** (primary influent, primary effluent, secondary effluent and clarified effluent). It should be noted that the secondary effluent contains activated sludge, which is separated by the clarifier. The measured wastewater parameters for secondary and clarified effluent indicated almost the same values. The reason for investigation of the samples from both treatment stages was to investigate the influence of the particles, which can not be separated by selected preparation methods, on test result. In this way, the efficiency of the used preparation method (see **Table 10**) can be further investigated.

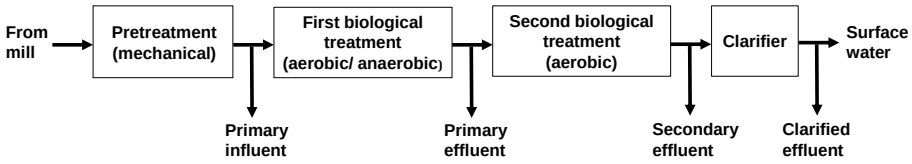


Figure 15: Sampling stages in each paper mill for multiple screening.

Wastewater samples were taken from paper mills with different products (corrugated boards, graphic papers, hygienic papers). Depending on the manufactured products and the composition of the wastewater, anaerobic-aerobic or two-stage aerobic wastewater treatment techniques are utilized in these paper mills. The Lemna minor test procedure in this investigation is based on the standardized method, which is described in chapter **6.6**. The investigated paper mills and related necessary information are summarized in **Table 6**.

Table 6: Investigated paper mills for multiple screening.

Source	Paper mill code	Paper type	Paper mill size	Wastewater treatment
Recovered paper without deinking	A	Corrugated board	Large	Anaerobic-aerobic
	B		Large	Anaerobic-aerobic
Recovered paper with deinking	C	Hygienic paper	Medium	Anaerobic-aerobic
	D		Medium	Anaerobic-aerobic
	E	Graphic paper	Large	Aerobic-aerobic
	F		Large	Aerobic-aerobic
	G		Large	Anaerobic-aerobic

5.6 Investigation of repeatability of the Lemna minor test results

Previous studies on the Lemna minor test using clarified effluent samples showed a large variation in the obtained test results, even with same sample and in the same laboratory. Moreover, in some investigations incomprehensible elevated toxicity values for effluent samples of graphic paper mills with the deinking process were observed. A significant factor for a variation in the test results in previous investigations may be different conservation conditions of the tested sample before test execution. Hence, the repeatability of the test results after standardization of the Lemna minor test procedure was further investigated in this work (see chapter **6.6**). For this purpose, the clarified effluent samples from seven paper mills were stored under described conditions in **Table 10**. Subsequently, the same sample from each paper mill was tested three times ranging from one day to two weeks after sampling under the same conditions. This study enables the estimation of the variability of the test results for the effluent sample of the same paper mill. Furthermore, the obtained G_w value for samples from mills with comparable paper type production, as well as samples which were treated by the same wastewater

treatment technique but in different paper mills, can be compared. The investigated paper mills in this section were identical to the previous part, summarized in **Table 6**.

5.7 Investigation of the correlation between test duration and result

The objective of this investigation is to identify, if the toxic effect of an effluent sample on Lemna minor can be evaluated in five days or if a minimum of seven days is necessary to obtain an accurate toxicity assessment.

The toxicity over time can be described by damage caused by a toxicant on a test organism in a defined period [105]. In this work, the effect of toxicants on test organisms is defined as growth inhibition of Lemna minor. For evaluation of the growth inhibition of Lemna minor over time, the clarified effluent samples from seven different paper mills were used for the test. The obtained results for growth inhibition as well as G_w value were then evaluated after two, five, and seven days of the experiment. Different dilution levels of the effluent samples including D1, D2, D4, and D8 were tested to examine the dependency of toxicant concentration on Lemna minor over time. To verify the experiment and to ensure the response of Lemna minor to toxicant over time, the reference samples containing 3.5 dicholorophenol (see **Figure 11**) were also tested at the same time and under the same conditions as effluent samples.

The Lemna minor test and sample preparation was performed under standardized conditions described in **Table 10**. The list of investigated paper mills for this section is summarized in **Table 6**.

6 Results and Discussion

6.1 Verification of the test validation

An example of Lemna minor growth in a control sample is shown in **Figure 16**. The exponential growth of Lemna minor can be observed from the start of experiment and increase continuously until the end, based on both observation parameters, growth number, and growth area.

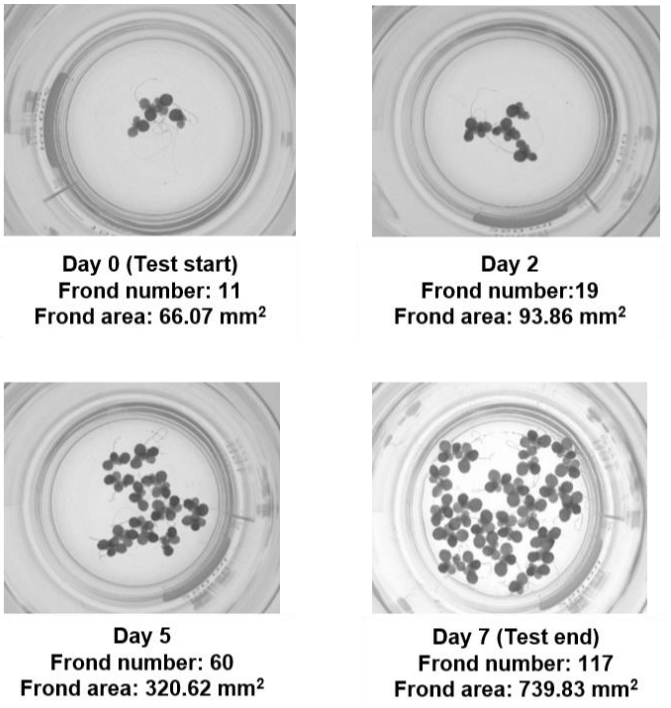


Figure 16: Exemplary evolution of Lemna minor growth in the control sample.

Figure 17 shows the mean frond numbers (absolute values) in the control approaches (n = 6). The error indication symbolizes the associated standard deviations for all control samples in the test series. The calculated standard deviation indicates a slight increase by the end of the experiment (day 7). This is caused by

biological growth, which is slightly different in each beaker despite the same test conditions.

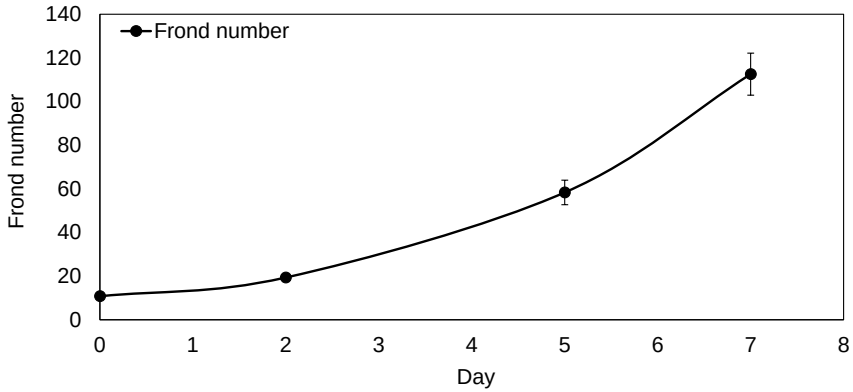


Figure 17: Exemplary trend of the mean values of the frond number for the control approaches over seven days.

According to the DIN ISO 20079:2006, the exponential growth of Lemna minors must be observed in control approaches. This can be verified considering the regression line (y) and its coefficient of determination (R^2). Respectively, **Figure 18** and **Figure 19** show the logarithmic growth of Lemna minor in seven days based on frond number and frond area.

The exponential growth of Lemna minor (based on frond number and frond area) within seven days of the experiment is evident in both diagrams. The calculated value of $R^2 = 0.99$ based on frond number and $R^2 = 0.98$ based on frond area proved the excellent fit of the mathematical linear regression with the physical growth of Lemna minor over the observation time. These two parameters demonstrate the exponential growth of Lemna minor in the control approaches within the test period. This was evaluated for each test series in this work to ensure the validity of the executed experiment.

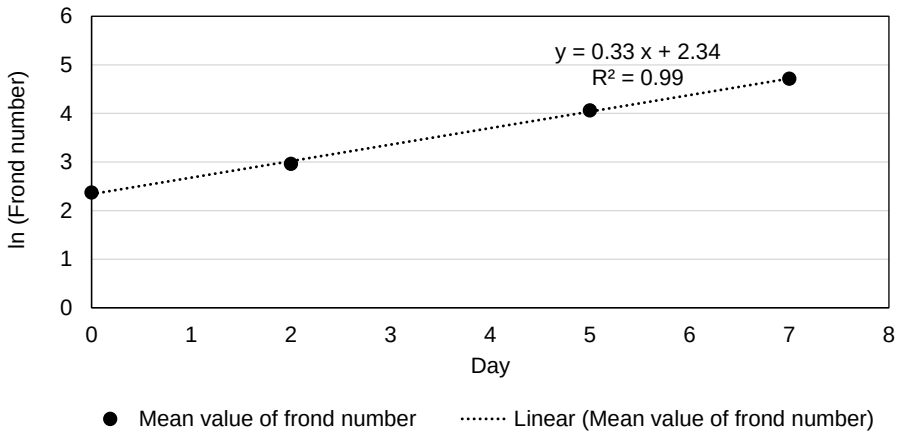


Figure 18: Illustration of the logarithmic mean value of frond number over the monitoring period.

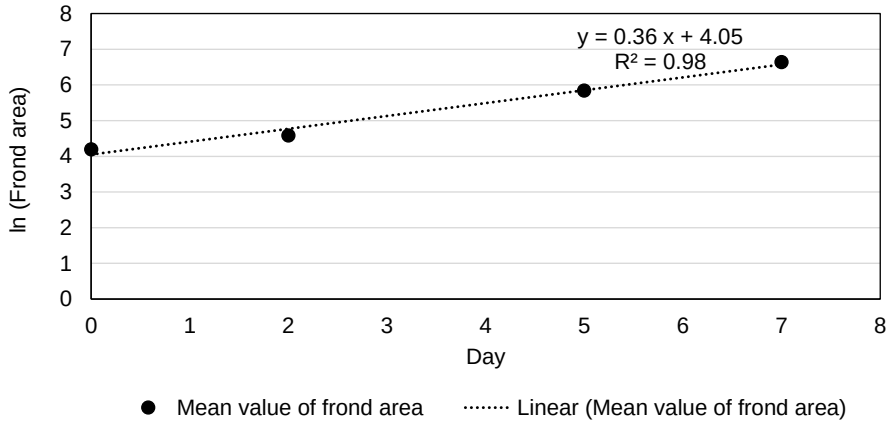


Figure 19: Illustration of the logarithmic mean value of frond area over the monitoring period.

The following validity criteria according to the DIN ISO 20079:2006 were fulfilled in the conducted tests within this work:

- The frond number in the control samples showed a mean specific growth rate of at least 0.275 per day. This corresponds to a doubling time of about 2.5 days and a 7-fold increase in the mean frond number at the end of the test.
- The EC_{50} based on frond number was reached for 3.5-dichlorophenol in reference approaches ranging from 2.2 mg/l to 3.8 mg/l.

For Lemna minor test that could not fulfill these validity criteria, the test was classified as invalid and repeated again.

The Lemna minor test for disintegrated paper sample (see **5.1**) was repeated three times under the same conditions and in three different floor levels of the plant growth cabinet. The growth inhibitions of all test samples including reference approaches based on frond number and frond area are demonstrated in **Figure 20**.

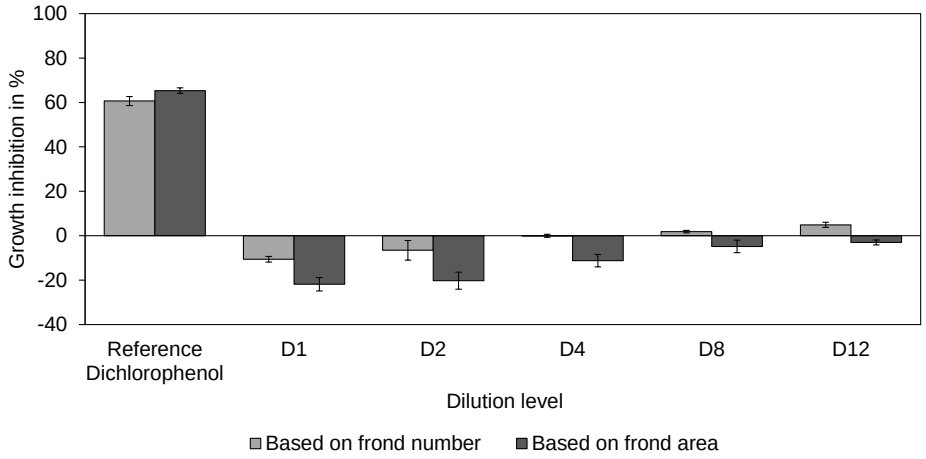


Figure 20: Growth inhibition of the test samples (D1, D2, D4, D8 and D12) compared to control approaches.

According to the DIN ISO 20079:2006, growth inhibition of less than 10 % relates to the harmless sample. A value greater than 50 % corresponds to the death of Lemna minor and is classified as a toxic sample for Lemna minor. Growth inhibition of higher than 10 % is not measured in any of the tested samples. The higher

dilution levels (lower sample concentration) indicated greater growth inhibition. Hence, Lemna minor growth rate was highest in the undiluted samples (D1). Based on the obtained results in **Figure 20** the tested disintegrated paper sample could not negatively influence the growth rate of Lemna minor, but also it contains substances and ingredients that could improve the growth of Lemna minor compared to control samples. These can be seen in **Figure 20**, as the growth inhibition of almost all dilution levels was negative, which corresponds to the better growth rate of Lemna minor in these samples compared to control approaches.

6.2 Influence of pH value and storage time

The Lemna minor test can not be executed on the sampling day in most cases, therefore the influence of each test parameter should be investigated over conservation time. This section first describes the influence of different pH values on the Lemna minor test. Subsequently, the change in growth inhibition for each adjusted pH is plotted over the preservation time. The objective is to define a pH value, which ensures the lowest change of growth inhibition over time for the same sample.

The growth inhibitions of Lemna minor for investigated pH values which ranged from 5.0 to 8.0 (tested pH values: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) using disintegrated paper samples are shown in **Figure 21**.

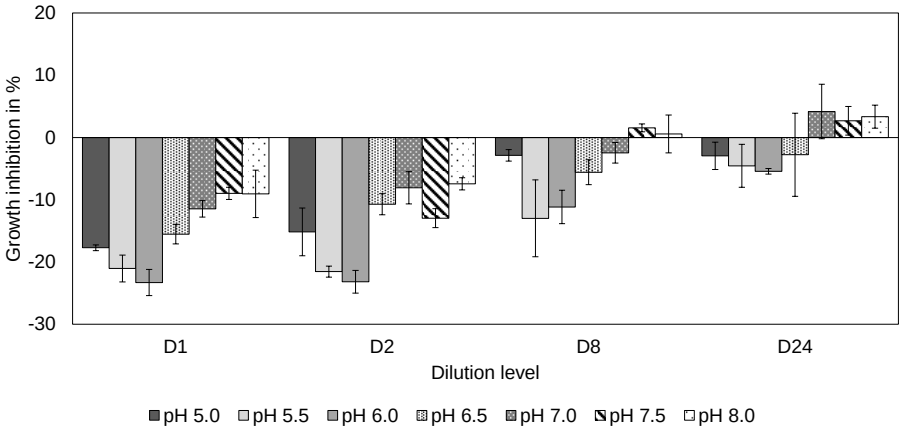


Figure 21: Growth inhibition of Lemna minor for different dilution levels of the disintegrated paper sample according to adjusted pH value after seven days (based on frond area).

According to DIN EN ISO, the pH adjustment for a sample with a pH value between 5.0 and 8.0 is not necessary, but adjusting the pH value of 5.5 for control and reference samples is mandatory [16]. The studies on the investigation of paper mills effluent using the Lemna minor test are performed by adjusting the pH value of 5.5 for all samples [1, 17]. However, nothing is known about the influence of pH value on the result of the test. Since the growth inhibition is calculated by comparing the

growth rate of Lemna minor in control samples (pH 5.5) and effluent samples, any effect of this parameter on growth rate can cause inaccuracy in test results.

As can be observed from **Figure 21**, adjusted pH values can influence the resulting growth inhibition. The lowest growth inhibition of Lemna minor is obtained with a pH value of 5.5 and 6.0 for all dilution levels. However, the difference of growth inhibition by changing pH value is greater for D1 and D2 with higher sample concentration. To have a better idea of the change in pH value of the samples over seven days of the experiment, **Figure 22** shows the measured pH values for undiluted samples with different adjusted pH values, including the control sample with an initial pH value of 5.5.

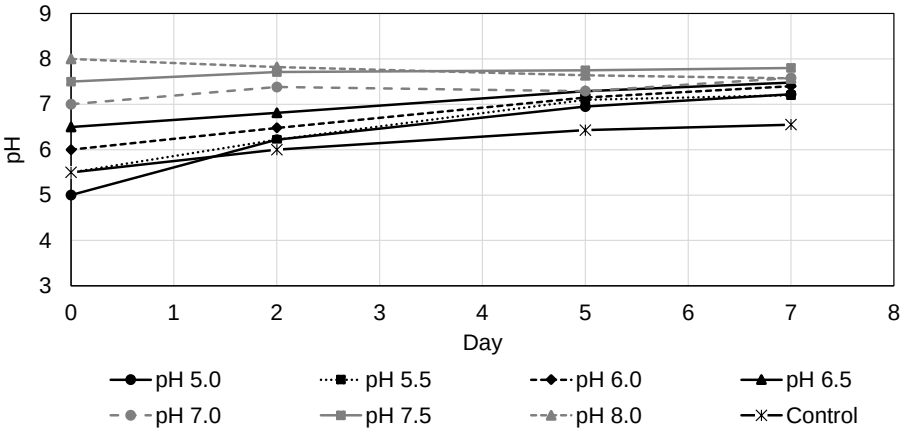


Figure 22: The change of adjusted pH values within the test period for the undiluted disintegrated paper sample (D1) and control sample.

The pH value of the samples does not remain constant over seven days. This change also occurred in control samples within seven days of the experiment (from 5.5 to about 6.5). The manual adjustment of the pH value can influence the absolute value in the first five days of the experiment. According to **Figure 22**, a significant change in pH value is observed in the first two days. This change decreased from day 2 to day 5 and remains almost constant from day 5 to day 7. It is obvious from **Figure 22** that the lower adjusted pH value resulted in greater change within seven days of the experiment. The pH value of the undiluted samples (D1), independent of the

adjusted pH value at the beginning of the test, indicates a value of 7.3 ± 0.2 at the end of the test. According to **Figure 21** and **Figure 22**, by adjusting the pH value of 5.5 and 6.0 at the beginning of the test better cultivation conditions for Lemna minor can be ensured. The purpose of adjusting the pH value is to provide the same test conditions for all investigated samples. The original pH value of the samples changed during seven days and can also negatively affect the growth rate of Lemna minor. Thus, the resulted growth inhibition is not only because of the toxic substances in the wastewater sample but is also because of the negative effect of pH value on plants. By providing the optimal growth zone, especially in the first days of the experiment, the negative effect of test conditions on results can be avoided.

In the next step, the influence of pH value on a real effluent sample is tested over time. The conserved clarified effluent samples from paper mill A under frozen conditions were defrosted at room temperature and sedimented for 1 h before the test. Subsequently, two test series by adjusting pH values of 5.5 and 6.0 were performed. The reason for testing these two pH values was to obtain a higher growth rate of Lemna minor in the previous investigation (see **Figure 21**). **Figure 23** and **Figure 24** illustrate the growth inhibition of Lemna minor for the tested sample over time, based on frond number and frond area for D2. Since the G_w value of lower than 10 % was first obtained by D2, the change of the growth inhibition over time was indicated for dilution level 2 in the following diagrams.

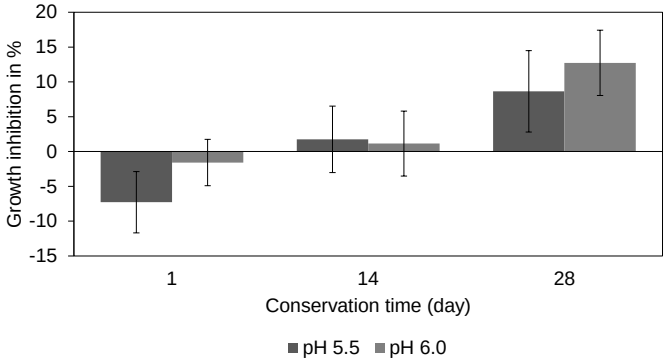


Figure 23: Influence of pH value on growth inhibition (based on frond number) over conservation time for D2 of clarified effluent of paper mill A.

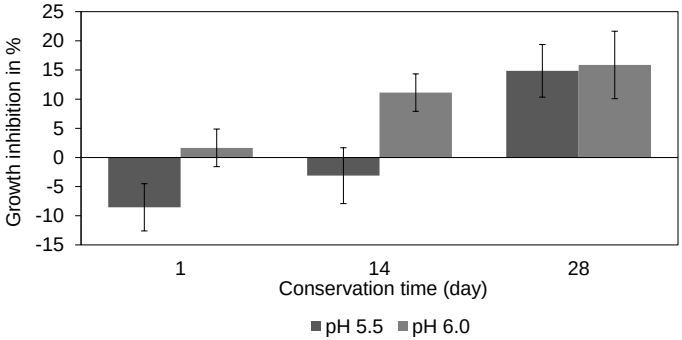


Figure 24: Influence of pH value on growth inhibition (based on frond area) over conservation time for D2 of clarified effluent of paper mill A.

Growth inhibition of Lemna minor for the clarified effluent sample with two different pH values indicated a similar trend over conservation time. However, the lowest change based on frond area within 14 days conservation time is obtained with pH 5.5. This can also be observed in **Figure 25** for the different dilution levels. It can be shown that the growth inhibition is lower for samples with higher dilution levels (correspond to lower effluent sample concentration). Additionally, the effect of conservation time and adjusted pH is not significant for D8 onwards. Although the pH of D1 to D4 changes from 5.5 during the 14-day conservation time, it remains below 10 %. For both adjusted pH values, a remarkable increase after 14 days can be observed. These also resulted in growth inhibition of higher than 10 % and classification of the sample as toxic.

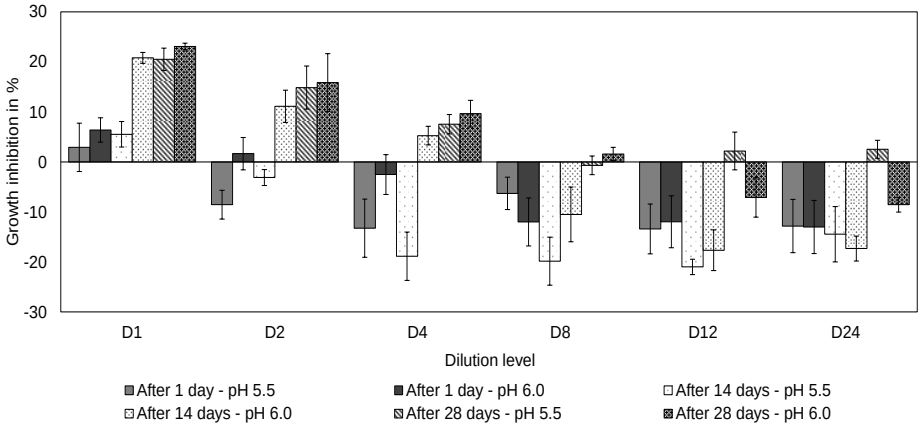


Figure 25: Growth inhibition (based on frond area) for different dilution levels of clarified effluent sample from mill A regarding to pH and conservation time.

Comparing the investigated pH values, a lower change in growth inhibition is observed over time by adjusting pH to 5.5. Furthermore, a pH value of 5.5 can provide a better growth condition for Lemna minor within the experiment. Therefore, adjusting the pH value of 5.5 for all samples including control and reference approaches is considered for standardization of the Lemna minor test procedure.

6.3 Influence of conservation temperature and storage time

To investigate the influence of conservation temperature on test results, clarified effluent samples from paper mill A were stored for different periods, ranging from one day after sampling to four weeks after sampling, at room temperature (24 °C), in the refrigerator (4 °C) and the freezer (- 18 °C). Subsequently, all samples were sedimented for 1 h and used for Lemna minor test. For investigated effluent samples, growth inhibition below the threshold of 10 % is achieved starting from dilution level 2 ($G_w = 2$) based on the frond number and frond area for all investigated temperatures. **Figure 26** and **Figure 27** present the obtained results for the change in growth inhibition of Lemna minor over time at different conservation temperatures for D2. For the frozen stored samples, the Lemna minor test was performed every two weeks. The studied conservation time for the samples stored at room temperature and in the refrigerator was based on the calculated growth inhibition. For these samples, the experiment was performed every week until the calculated growth inhibition reached a value of ≥ 10 % either based on frond number or frond area.

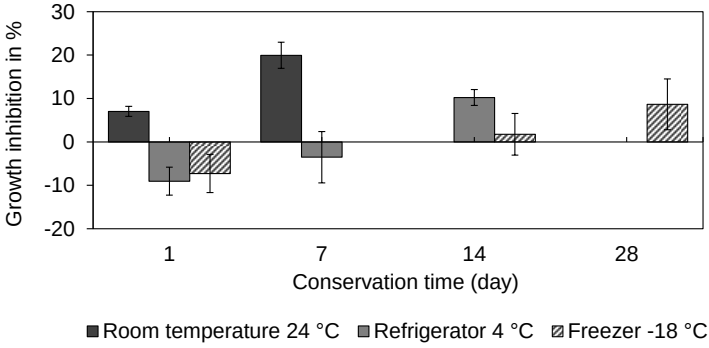


Figure 26: Influence of the conservation temperature on growth inhibition (based on frond number) over time for D2 from clarified effluent of paper mill A.

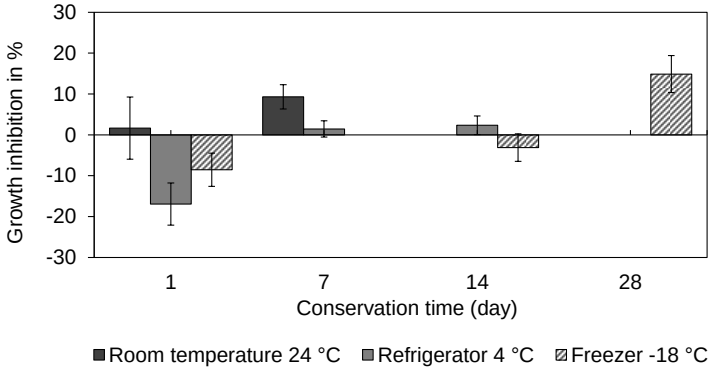


Figure 27: Influence of the conservation temperature on the growth inhibition (based on frond area) over time for D2 from clarified effluent of paper mill A.

The stored effluent samples at room temperature indicate a significant increase in growth inhibition over time, so that growth inhibition for a nontoxic sample (< 10%) is higher than 10 % after seven days of conservation at room temperature. A similar trend is also observed for the conserved effluent samples in the refrigerator in the period from one day to seven days after sampling based on the frond area. This is followed by only minor changes between seven and fourteen days. For the stored samples in the refrigerator, a different trend is recorded based on the frond number. These indicate a minor shift in growth inhibition up to seven days after sampling. In the further investigation period of seven to fourteen days of storage, there is a significant increase in growth inhibition. As the growth inhibition of Lemna minor after seven days of conservation in the refrigerator is still below 10 % based on frond area and even in the negative range based on frond number, it can be concluded that the conservation of the effluent sample up to seven days in refrigerator has no significant negative effect on sample properties. The smallest temporal change in growth inhibition is achieved with conserved effluent samples in the freezer for up to fourteen days. If the storage time is extended to 28 days, a significant increase in growth inhibition occurs. **Figure 28** shows the linear relationship of growth inhibition over conservation time for investigated temperatures based on frond number.

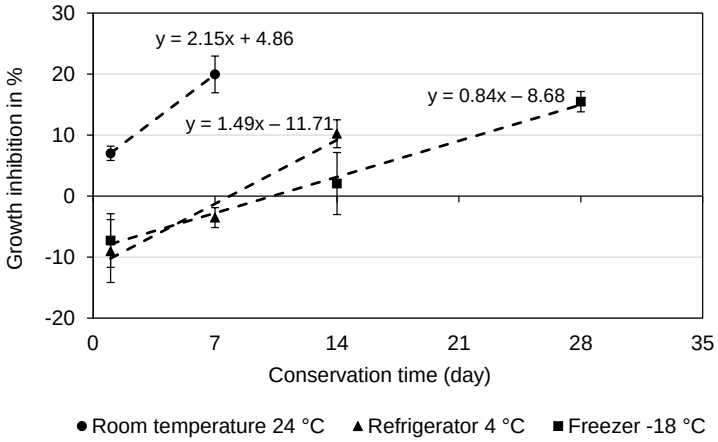


Figure 28: Correlation between growth inhibition (based on frond number) and conservation time for different conservation temperatures.

A distinct correlation between growth inhibition and storage time can be observed in **Figure 28**. The conservation of effluent samples at room temperature results in the highest initial growth inhibition and the most significant change over time, whereas the lowest effect is obtained for frozen samples. A storage time longer than fourteen days is also not recommended, even for frozen samples, as growth inhibition increases significantly after this time.

6.4 Influence of sample preparation method and storage time

This chapter first describes the significance and influence of the separation of solid and colloidal dissolved substances from the effluent sample on the test results. **Figure 29** presents the obtained results for Lemna minor growth inhibition using clarified effluents of three paper mills, directly after sampling and one week after sampling (storage under frozen conditions). The frozen wastewater samples were defrosted at room temperature one day before the start of the test and homogenized in a 1 l beaker using a magnetic stirrer. The values assigned to the columns correspond to the TSS value of the tested clarified effluent samples.

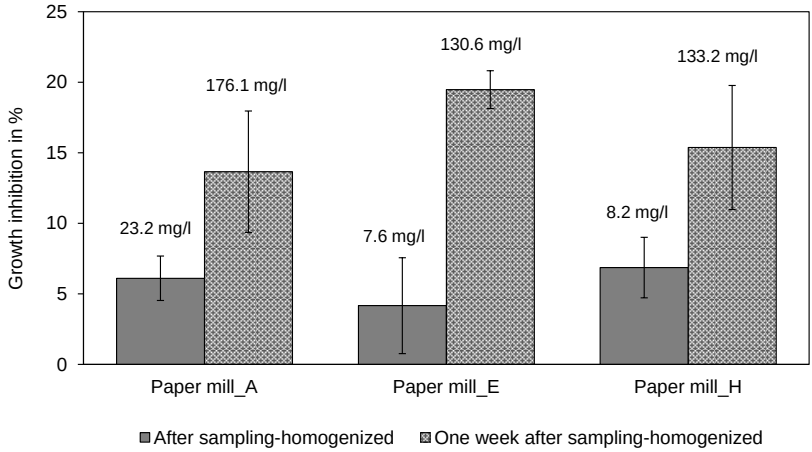


Figure 29: Growth inhibition (based on frond area) of the homogenized clarified effluent samples, directly and one week after sampling for three different paper mills.

The TSS of the wastewater samples is considerably increased after one week of storage in the freezer. This value was less than 25 mg/l for all three samples directly after sampling. Freezing of the samples leads to a significant increase in the TSS value (> 120 mg/l), which negatively influences the growth inhibition of Lemna minor.

To compare the effect of TSS value on growth inhibition, the same sample was filtered via black ribbon filter paper after one week of storage under frozen conditions. **Figure 30** compares the calculated growth inhibition for homogenized and filtered clarified effluent samples after one week of storing in the freezer with the same homogenized sample, which was tested directly after sampling.

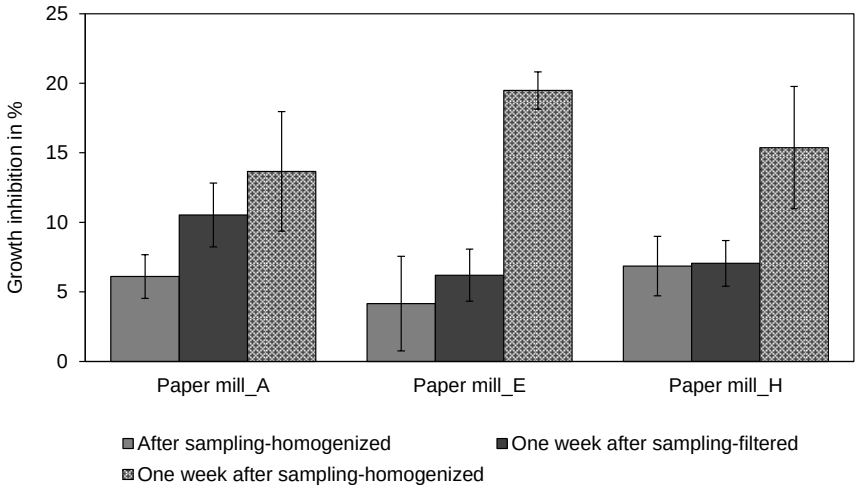


Figure 30: Comparison of the growth inhibition of the homogenized and filtered clarified effluent sample after one week of storage in the freezer for three different paper mills.

According to **Figure 30**, lower growth inhibition is obtained with the filtered effluent sample compared to the homogenized sample after one week of storage under frozen conditions. The growth inhibition of the filtered samples after freezing is almost identical to the growth inhibition of the sample tested directly after sampling. It should be considered that unsuitable storage conditions and preparation methods can significantly affect the properties of the effluent sample in a way that a harmless sample is classified as a toxic sample. Among different preparation methods such as sedimentation, centrifugation and filtration, the method which results in minimum variation in growth inhibition over storage time should be selected. For this purpose, clarified effluent samples from three different paper mills (mills A, E and H) were stored under frozen conditions for a period of one day to four weeks after sampling. Samples were prepared according to the defined conservation time using four different methods described in chapter **5.4.1**. The major objective of this investigation is to evaluate the impact of the solids of different particle sizes present in the effluent sample on growth inhibition. The obtained results are shown in the following **Figure 31 A-C**.

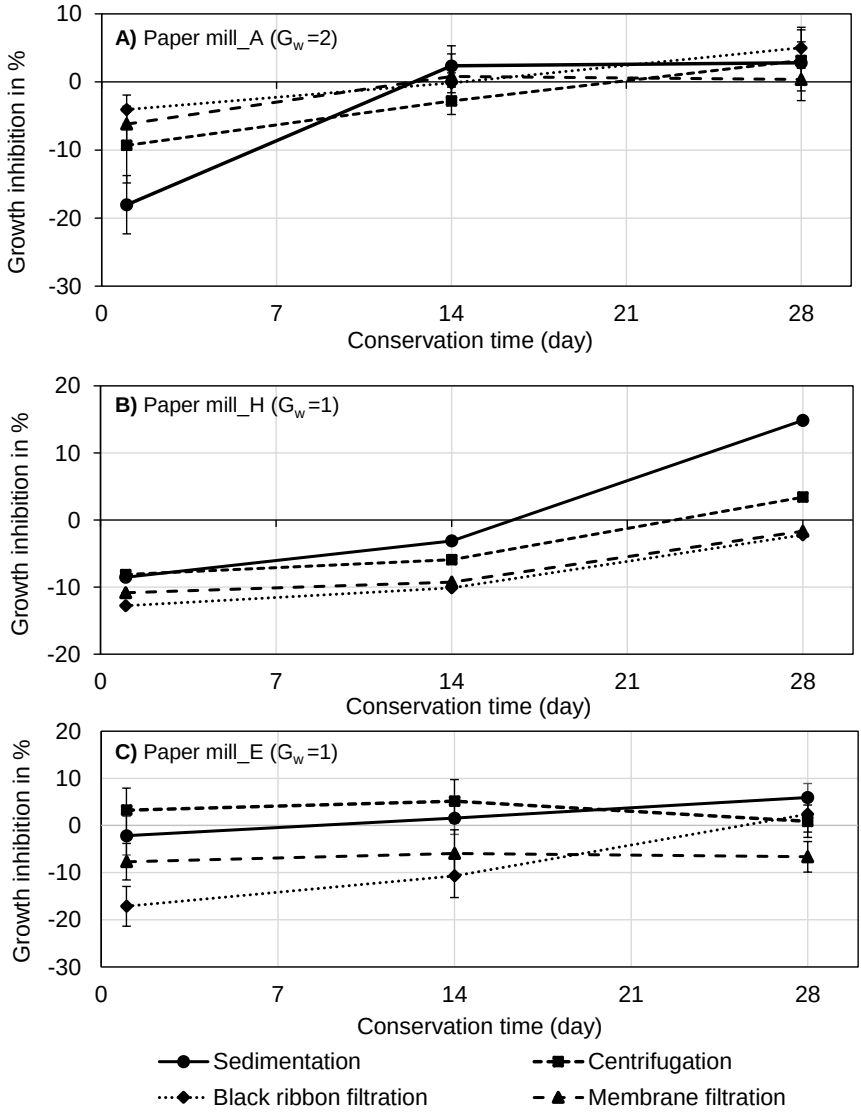


Figure 31: Influence of different preparation methods on growth inhibition of Lemna minor (based on frond area) by testing clarified effluent samples from three paper mills.

The filtered effluent samples via black ribbon filter paper from paper mill A and E indicate a negligible change in growth inhibition over time. Sedimented effluent samples from mill A and H (**Figure 31 A** and **B**) show a high change in growth inhibition within one week (mill A), or from the first week to the second week (mill H). The variation in growth inhibition for the centrifuged effluent samples resulted in different trends for the investigated paper mills. Filtration of the sample by black ribbon filter for the effluent from paper mill A and E resulted in only minor alteration of the growth inhibition over time. Satisfactory results were also achieved for paper mill H with this preparation method. Almost the same results are obtained for filtration via black ribbon and membrane filter in all investigated paper mills. Moreover, these two methods result in a minor change in growth inhibition over storage time. According to the retention capacity of both filter (black ribbon 7-12 µm, membrane filter 0.4 µm), it can be conducted that particle size smaller than 7 µm can not remarkably influence the result of the Lemna minor test. Furthermore, the obtained result of growth inhibition for the filtered sample via black ribbon filter paper is almost identical to the growth inhibition of the sample directly after sampling (see **Figure 30**). Therefore, the filtration of the sample via black ribbon filter paper is selected and used as a preparation method for further investigations in this work.

Similarly to the obtained results in sections **6.2** and **6.3**, also in this part the storage time of longer than 14 days causes a noticeable change in growth inhibition. Hence, the time between sampling and test execution has to be reduced to maximum of 14 days.

6.5 Required number of dilution levels for Lemna minor test

The determination of the NOEC is described in this section using an example of the investigated clarified effluent sample. The NOEC calculation for all tested samples is demonstrated in detail in **Appendix I**. According to the obtained results in **6.3** and **6.4**, the sample used for Lemna minor test can only be conserved for up to two weeks under frozen conditions and must be filtered via black ribbon filter paper before to the test. Based on that, the NOEC was calculated for the clarified effluent samples, which were tested under mentioned conditions. The ANOVA method is used to determine the NOEC. Here, the mean specific growth rate and the residual

standard deviation over the replicates are calculated for each test concentration. The resulting mean for each test concentration is then compared to the mean of control samples using Dunnett's test as a suitable multiple comparison method according to OECD guidelines for Lemna minor growth inhibition test [104].

Table 7 lists the raw data for the growth rate of Lemna minor in three samples of each dilution level based on frond area for paper mill H. The results for the ANOVA test are then reported in **Table 8**.

Table 7: Lemna minor growth rate (based on frond area) for different dilution levels incl. control samples for clarified effluent of paper mill H.

Sample	Control	D1	D2	D4	D8	D12	D24
Growth rate 1/day (based on frond area)	0.321	0.391	0.333	0.352	0.365	0.361	0.360
	0.315	0.364	0.352	0.346	0.376	0.388	0.372
	0.317	0.313	0.370	0.356	0.381	0.363	0.374

Table 8: Results of Anova test for clarified effluent of paper mill H.

α =0.05	Difference between groups	Difference within groups
Sum of squares (SS)	0.006	0.004
Degree of freedom	6	14
Mean sum of squares	0.001	0.0003
F-value (F statistic)	3.33	
P-value	0.029	
Critical F-value	2.84	

The Dunnett's test is calculated according to equations (3) and (4) (see **Appendix I**). The critical value for Dunnett's test (d = 2.91) can be determined according to the degrees of freedom and the sample size (number of groups) [106].

The minimum significant difference MSD (in this example = 0.029) can be calculated using the ANOVA test parameters and d value (see **Appendix I**). The t_i-value

describes the difference between the mean of the control group and the mean of each dilution level group. If the t_i-value calculated for the dilution level group is greater than MSD-value, this distance is significant, which relates to a significantly higher growth rate of Lemna minor in the defined dilution level group compared to the control samples. **Table 9** summarizes the calculated parameters for Dunnett's test.

Table 9: Results of Dunnett's test for clarified effluent of paper mill H.

Sample	Mean value	t_i	$t_i > MSD$(=0.029)
Control	0.379		
D1	0.406	0.026	No
D2	0.386	0.006	No
D4	0.407	0.028	No
D8	0.414	0.034	Yes = NOEC
D12	0.410	0.030	Yes
D24	0.412	0.032	Yes

The calculated t_i-value is higher than MSD-value (MSD = 0.029) from D8. From D8, the growth rate at each dilution level is as good as the control samples, and no toxic effect on Lemna minor is observed.

In three out of six tested samples, the NOEC was by D8 and the rest by D4 or D2 based on two observation parameters. Therefore, no toxic effect related to the effluent sample can be observed on Lemna minor from D8. For this reason, the Lemna minor test for the clarified effluent samples can be performed with four dilution levels (D1, D2, D4 and D8).

6.6 Standardization of the Lemna minor test procedure

Based on obtained results in chapter **6.2** - **6.5** the standardized Lemna minor test procedure can be described as follows:

For toxicity assessment using Lemna minor test approximately 1.5 l of paper mill clarified effluent is required. Sampling can be performed either by laboratory personnel or by instructed personnel in the paper mill. After sampling, the effluent sample must either be analyzed directly or be frozen. The clarified effluent sample can be stored up to two weeks under frozen conditions. If sampling is executed by paper mill personnel, the effluent sample has to be shipped in frozen conditions and a plastic bottle as soon as possible (overnight shipment). The Lemna minor test must be carried out directly after receiving the effluent sample. A refreezing of the effluent sample is not recommended under any circumstances.

The effluent samples must be filtered via black ribbon filter paper before starting the test. For examination of the clarified effluent, Lemna minor test can be carried out with four dilution levels (D1, D2, D4, D8). The pH value of the samples including control and reference approaches must be adjusted to 5.5. The test procedure as well as the evaluation of the test results is carried out according to DIN EN ISO 20079:2006. The image analysis software applied has to be accurate enough to ensure reliable results.

It is recommended to keep the Lemna minor culture in a separate room and to change the nutrient medium every five days. To avoid possible contamination following points should be considered and executed during the test phase:

- Heating out and disinfection of the plant growth cabinet for at least two hours after finishing each test procedure.
- Heating out and disinfection the used beakers and the other equipment at 120 °C after finishing each test procedure.

The standardized Lemna minor test procedure is summarized in **Table 10**.

Table 10: Standardized Lemna minor test procedure.

Sampling – Sampling by instructed personnel. – Taking of approx. 1.5 liter effluent sample in plastic bottles, so that max. three quarters of the volume of bottles are filled with samples. Partial filling provides efficient empty space for volume expansion when the sample is frozen. – Freezing the effluent sample directly after collection, if the test cannot be executed on the same day. – For freezing the effluent sample at least 8 hours is required. – The samples have to be sent in frozen form and by overnight express delivery from the paper mill to the testing laboratory.
Storage conditions in the laboratory – Immediate freezing of the sample after receipt, if testing is not possible (it is only valid if the samples are still in a completely frozen state). – Storage of the sample under frozen conditions (-18 ± 2 °C) for a maximum of two weeks before testing can be considered.
Sample preparation method – The sample must be defrosted at room temperature one day before the start of the test (in the case of having a frozen sample). – Filtration via black ribbon filter paper before starting the test.
Test procedure – Test procedure according to DIN EN ISO 20079:2006. – Adjustment of the pH value to 5.5 ± 0.2 for all samples including control and reference approaches. – Lemna minor test can be executed with four dilution levels (D1, D2, D4, D8).

6.7 Multiple screening in paper mills

From previous studies, it is known that higher G_w values were obtained for paper mills equipped with an anaerobic-aerobic wastewater treatment system [5, 6]. Hence, five paper mills equipped with an anaerobic-aerobic and two paper mills equipped with an aerobic-aerobic wastewater treatment plant were investigated in this work. Within this investigation, wastewater samples were collected and tested from the following stages in each paper mill (see **Fehler! Verweisquelle konnte nicht gefunden werden.**):

- Primary influent (inflow of first biological stage)
- Primary effluent (outflow of the first biological stage)
- Secondary effluent (outflow of the second biological stage)
- Clarified effluent (outflow of clarification plant)

The investigation of clarified effluent samples was carried out by four dilution levels (D1, D2, D4 and D8) (see chapter **6.6**). For the samples from other treatment stages, D12 and D24 were additionally tested.

Figure 32 - **Figure 34** show the results of the Lemna minor test for the wastewater samples from investigated paper mills. For a better comparison of the test results, the calculated growth inhibitions for the paper mills producing comparable paper types are indicated separately in each diagram. For each investigated sample, the first dilution level, which indicates the growth inhibition below 10 %, is shown in the diagram. The values assigned to the columns correspond to the G_w value of tested wastewater samples.

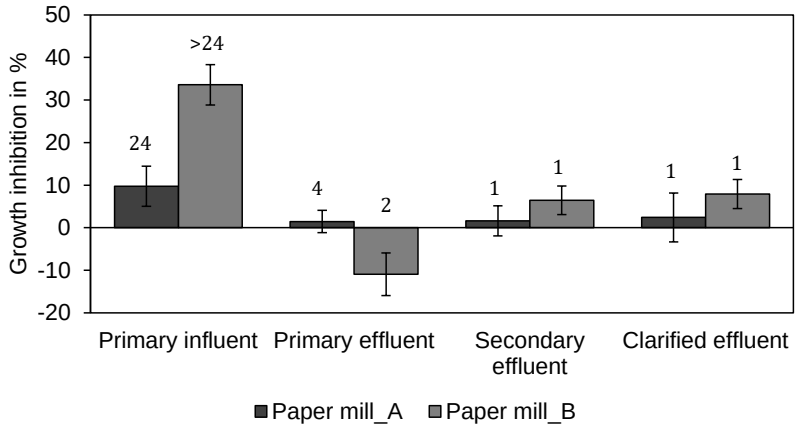


Figure 32: Growth inhibition (based on frond area) for wastewater samples from paper mills producing corrugated board at four different treatment stages.

Figure 32 shows the growth inhibition for the wastewater samples from corrugated board producers. These two investigated mills are equipped with an anaerobic-aerobic wastewater treatment plant. Despite the identically produced paper type in these mills, a higher G_w value is calculated for the untreated wastewater sample (primary influent) of mill B. The G_w value decreases significantly in paper mill B after the first and second biological stages, so that clarified effluent could achieve a growth inhibition of under 10 % for undiluted samples (D1). The same trend of reducing the G_w value is also observed for paper mill A. Despite a high G_w value for primary influent samples, the growth inhibition limit of 10 % is not exceeded for secondary effluent as well as clarified effluent samples even in undiluted samples (D1).

A high G_w value is obtained for samples from all tested treatment stages of paper mill C, indicated in **Figure 33**. This value is above 24 for primary influent and primary effluent. The determined G_w value for clarified effluent of mill C is also significantly higher compared to other paper mills having an anaerobic-aerobic treatment plant. Paper mill D shows a similar trend as paper mills A and B so that G_w value of 1 was obtained for the secondary effluent and clarified effluent samples.

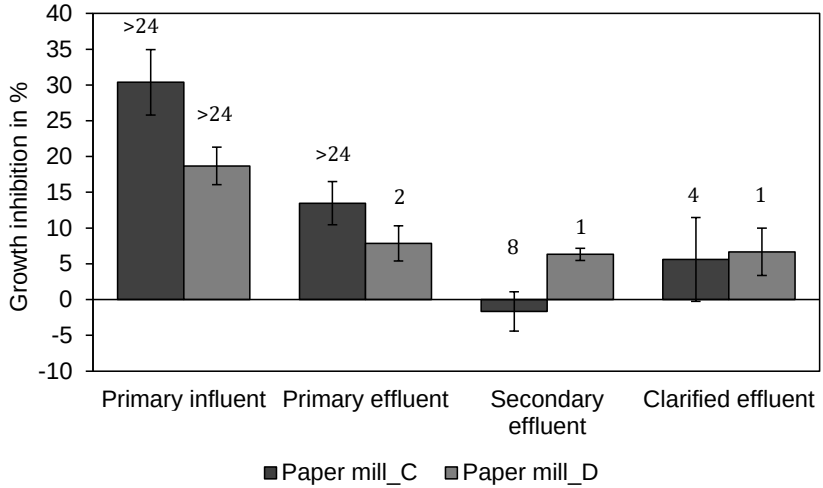


Figure 33: Growth inhibition (based on frond area) for wastewater samples from paper mills producing hygienic paper at four different treatment stages.

Figure 34 shows the growth inhibition for wastewater samples from graphic paper manufacturers. According to **Figure 34**, considerably lower G_w values are calculated for primary influent samples from paper mills producing graphic paper ($8 \leq G_w \leq 12$) compared to primary influent samples of corrugated board and hygiene paper manufacturers ($G_w \geq 24$). The evident difference between calculated growth inhibitions for graphic paper mills treating with aerobic-aerobic process (mill E and F) and paper mill G using an anaerobic- aerobic treatment process can be observed in **Figure 34**. Here, the highest G_w value for primary effluent, as well as clarified effluent samples, belong to mill G. At paper mills E and F the growth inhibition of lower than 10 % was observed in D1 of clarified effluent samples. The

calculated growth inhibitions for wastewater samples from mill E excluding primary influent are in the negative range, indicating a better growth rate of Lemna minor in these samples compared to control approaches. The fact is confirmed that some nutrients present in wastewater can also improve the growth rate of Lemna minor. However, this effect cannot be proven by the measured chemical and physical parameters of the investigated samples in this study.

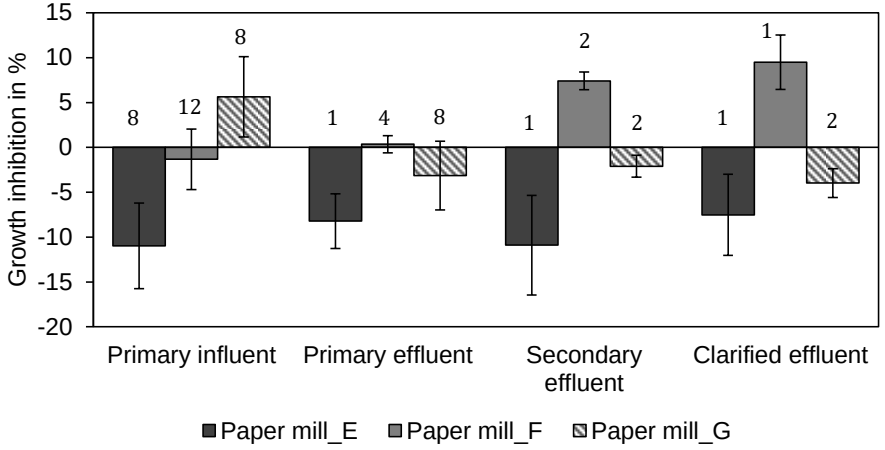


Figure 34: Growth inhibition (based on frond area) for wastewater samples from paper mills producing graphic paper at four different treatment stages.

The change in G_w value for an undiluted sample (D1) from primary influent to clarified effluent is shown in **Figure 35** for the investigated paper mills having anaerobic-aerobic and in **Figure 36** for paper mills treating with aerobic-aerobic wastewater technique. Wastewaters from the same produced paper type are labeled in the diagram with the same color and different symbols/lines. Paper mill G is shown in both diagrams because it is classified as anaerobic-aerobic in terms of wastewater treatment technology. On the other hand, it produces the same paper type as mills E and F, which have an aerobic-aerobic wastewater treatment process.

Both figures provide an overview of the reduction in growth inhibition of Lemna minor along the wastewater treatment process. For paper mills A, D and G, the continuous reduction in growth inhibition can be observed from primary influent to secondary effluent, while for paper mills B and C a considerable reduction of growth inhibition is shown by the first biological treatment. Almost the same growth inhibitions for secondary effluent and clarified effluent are obtained for all investigated paper mills. Except for mill C, the G_w value of all investigated clarified effluent samples is 1 or 2. It can be concluded that the currently used wastewater treatment techniques in paper mills can significantly reduce the G_w value.

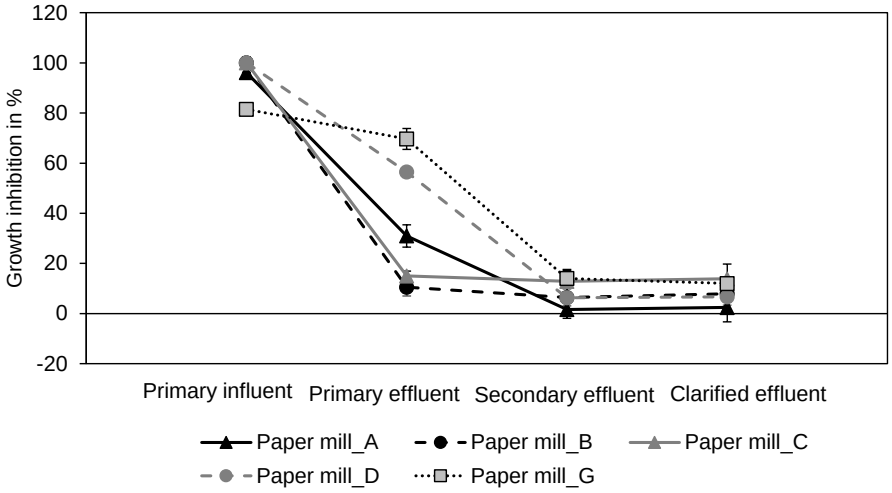


Figure 35: Monitoring the growth inhibition along wastewater treatment process for D1 samples of paper mills using anaerobic-aerobic treatment.

According to **Figure 36**, the highest growth inhibition belongs to the primary influent of paper mill G. Even after the first biological treatment stage (primary effluent), there is still a significantly high level of growth inhibition, remaining above mills E and F. A slightly higher growth inhibition is obtained for clarified effluent sample of mill G compared to mill F. However, this results in a different G_w value for clarified effluent of mills F (G_w = 1) and G (G_w = 2). Paper mill E has a substantial reduction in growth inhibition after the first biological treatment stage and then

remains almost constant until the end of the treatment process. Whereas a stepwise reduction of growth inhibition is observed for mill F and G.

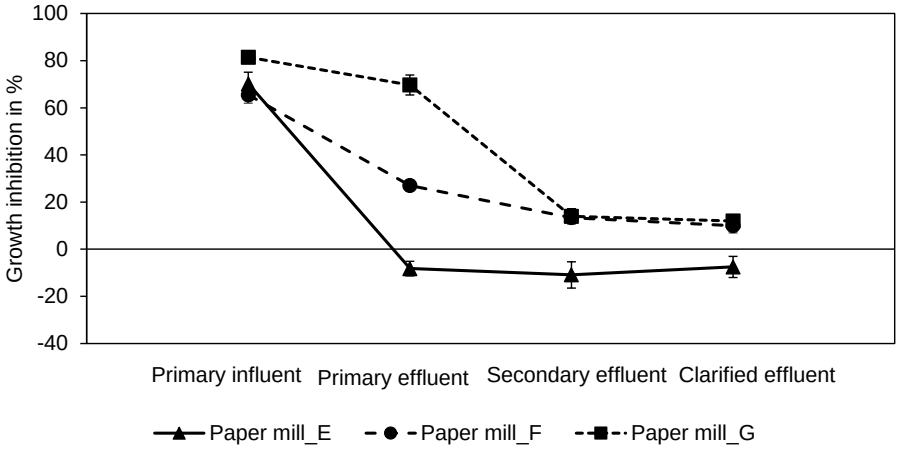


Figure 36: Monitoring the growth inhibition along wastewater treatment process for D1 samples of paper mills using aerobic-aerobic treatment.

The different reduction trends of growth inhibition along the wastewater treatment process in the investigated paper mills could not be reasonably explained by the provided information in questionnaires of each paper mill. However, the measured wastewater parameters can mainly explain the difference between growth inhibitions of wastewater samples in each treatment stage. This is discussed in detail in chapter **6.8**. It should be noted that the exact amount of utilized substances (e.g. defoamers, flocculants, etc.) in each paper mill and in the sampling time could not be recorded. In addition, the comparison of utilized wastewater treatment in each paper mill was only based on treatment techniques (anaerobic or aerobic process). While the reactor types, adjustments and detailed processing in paper mills were not considered in this study.

In contrast to previous studies, there was no increase in growth inhibition for paper mills with a deinking plant as well as after the aerobic stage observed in this study. The clear trend over the dilution levels was recognizable for all investigated samples. The obtained G_w range for treated samples with the anaerobic-aerobic

process was higher ($1 \leq G_w \leq 4$) than treated samples with aerobic- aerobic techniques ($G_w \leq 2$), but still considerably lower than reported results ($2 \leq G_w \leq 8$) in previous investigations [1].

6.8 Influence of wastewater parameters on the test result

In this chapter, the influence of chemical and physical wastewater parameters on Lemna minor test results is investigated and discussed. For this purpose, the collected wastewater samples were analyzed before the start of the test and after filtration via black ribbon filter paper. The Lemna minor test was performed according to **Table 10**. The measured chemical and physical parameters for investigated samples from different treatment stages in paper mills including the resulted growth inhibition are indicated in **Table 11** and **Table 12**.

Table 11: The measured chemical and physical parameters including resulting growth inhibition for investigated primary influent and primary effluent samples (D1) from seven paper mills.

Parameter		COD mg/l	AOX mg/l	Turbidity FTU	TN_b mg/l	P_{tot} mg/l	surface tension mN/m	i_r_A %
Mill code								
Primary influent	A	3034	0.39	192	15	2.75	72.25	96.13
	B	3200	0.27	180	17	13.8	-	100
	C	3089	0.38	92.4	23	2.35	-	100
	D	4743	0.46	95	89	1.75	-	100
	E	1715	0.06	32.1	6	0.65	71.93	70.15
	F	1892	0.11	28.1	12	1.2	73.11	63.46
	G	1802	0.12	40	14	0.8	-	81.45
Primary effluent	A	1320	0.33	185	21	7.35	73.45	30.95
	B	804	0.15	170	19	3.65	-	10.53
	C	668	0.32	130	15	2	-	9.98
	D	1176	0.43	80	48	0.95	-	56.45
	E	912	<0.05	15	13	0.6	74.25	-8.22
	F	808	0.08	16.4	13	7.65	73.05	26.99
	G	1038	0.08	18.10	15	0.6	-	69.68

Table 12: The measured chemical and physical parameters including resulting growth inhibition for investigated secondary effluent and clarified effluent samples from seven paper mills.

Parameter		COD mg/l	AOX mg/l	Turbidity FTU	TN_b mg/l	P_{tot} mg/l	surface tension mN/m	i_r_A %
Mill code								
Secondary effluent	A	266	0.28	2.27	18	0.8	72.83	1.61
	B	252	0.11	1.74	10	0.9	-	6.43
	C	209	0.17	1.38	7	2.5	-	12.88
	D	230	0.14	1.6	7.4	0.6	-	6.32
	E	363	<0.05	1.7	9	0.8	73.15	-10.9
	F	247	0.06	0.23	7	0.5	74.25	13.4
	G	249	0.08	0.27	7.3	0.2	-	14.02
Clarified effluent	A	261	0.23	2.23	17	1.05	71.96	2.42
	B	240	0.13	1.22	10	0.45	-	7.92
	C	224	0.17	0.96	7.2	0.35	-	13.86
	D	180	0.12	3.16	8.9	0.25	-	6.67
	E	330	<0.05	1.2	6.1	0.24	71.88	-7.52
	F	236	0.09	0.29	6.8	1.9	71.27	9.53
	G	244	0.08	0.26	7.1	0.2	-	12.11

The significant difference between measured COD values for primary influent samples from different paper mills can be observed in **Table 11**. This value ranges from ≈ 1900 mg/l to about 4700 mg/l. Factors influencing the COD value of paper mills wastewater can include the used raw materials, chemical additives, specific wastewater volume as well as internal water purification and recycling systems [8]. The high COD load in paper mills A and B is primarily a result of the deficiencies in the water recycling system. Additionally, the use of large quantities of strength-enhancing chemical additives, especially starch is another reason for the high COD value in these paper mills [8]. A significantly high COD value also results from the

untreated wastewater of hygienic paper productions (mills C and D). These paper mills utilize recovering paper as a raw material. Due to the quality requirements for hygienic paper products, stronger bleaching of the raw material is needed, which results in a higher COD value in their wastewater [59]. However, the COD value in all investigated paper mills is reduced significantly along the wastewater treatment process and remains under 350 mg/l for clarified effluent samples.

Sources of absorbable organic halides (AOX) in wastewater of paper mills can be natural halogen compounds contained in wood, as well as substances from bleaching with chlorine-containing chemicals, chemical additives, or dyes [28]. The higher AOX values are measured for the primary influent samples from corrugated board and hygienic paper production. These paper mills typically use a larger quantity of chemical additives. In addition, the stronger bleaching process for the production of hygienic papers causes a higher AOX value in primary influent samples. Since the anaerobic treatment process is not able to efficiently reduce the AOX content (see **2.3.1**), the high AOX values can be observed in primary effluents of paper mills using anaerobic-aerobic treatment (mills A, B, C and D). However, after aerobic treatment and in clarified effluent samples, AOX levels of less than 0.25 mg/l are observed for all paper mills.

The primary influent and primary effluent samples are also characterized by their high turbidity values, which is mainly due to the presence of insoluble matters and also soluble color compounds [107]. According to the measured values, higher turbidity can be obtained for wastewater samples from corrugated board and hygienic paper productions. A significant reduction of turbidity (less than ≈ 3.5 FTU) after aerobic treatment can be observed for all investigated paper mills.

The nitrogen and phosphorus content in wastewater of paper mills is primarily a result of the additives used in different stages of the production process. Furthermore, the nutrients used for the biological treatment of wastewater cause an increase in nitrogen and phosphorous content of wastewater in different treatment stages [28]. The measured values for TN_b and P_{tot} of clarified effluent samples from all investigated paper mills are below the limit values given by annex 28 (limit values: TN_b 20 mg/l and P_{tot} 2 mg/l) [10].

The reason for measuring surface tension in this investigation is the fact that Lemna minor grows on the water surface and thus any change in surface conditions can influence the growth of Lemna minor. The chance that small amounts of

defoamers and the other surfactants remaining in the wastewater sample after or during wastewater treatment affect the surface tension makes it essential to determine the surface tension of the samples before testing. The measured surface tension values for samples of different treatment stages from three paper mills (A, E and F) indicate only a minimal change and can not affect the growth rate of Lemna minor.

A toxicity test can be used to investigate the influence of each individual chemical and/or physical wastewater parameter, but also the interaction of different parameters on the test organism. In this study, all investigations are conducted using real wastewater samples with different parameters. Even samples from the same paper mill can deviate in parameters each time they are sampled. These are not only limited to the measured chemical and physical parameters in this work, but also in other parameters not considered here, as well as the interaction of different parameters on Lemna minor. Therefore, for the evaluation of the effect of chemical and physical parameters on the growth inhibition of Lemna minor, no clear correlation between the measured values and the determined growth inhibitions can be stated. Still, the following statements can be concluded from the obtained results:

- Investigation of primary influent and primary effluent samples results in higher values of COD, AOX, and turbidity. These higher levels of wastewater contaminations are associated with higher growth inhibition. Whether ultimately one of these factors or an interaction of several factors is responsible for the higher growth inhibition could not be clarified in this investigation.
- The expected positive effect of a higher concentration of nitrogen and phosphorus compounds in the primary influent and primary effluent samples on the growth rate of Lemna minor is overlaid by other effects and could not influence growth inhibition.
- The surface tension of the wastewater samples is not a relevant influencing factor for Lemna minor growth inhibition in this investigated area.

– For the seven investigated clarified effluents there are no significant correlations between the growth inhibition of Lemna minor and the investigated parameters COD, AOX, turbidity, nitrogen, and phosphorus content. This can be observed from **Figure 37**, indicating the influence of individual parameters on growth inhibition of Lemna minor. It can be conducted that the investigated parameters in the measuring range for clarified effluent samples can not be the influencing factor for growth inhibition of Lemna minor.

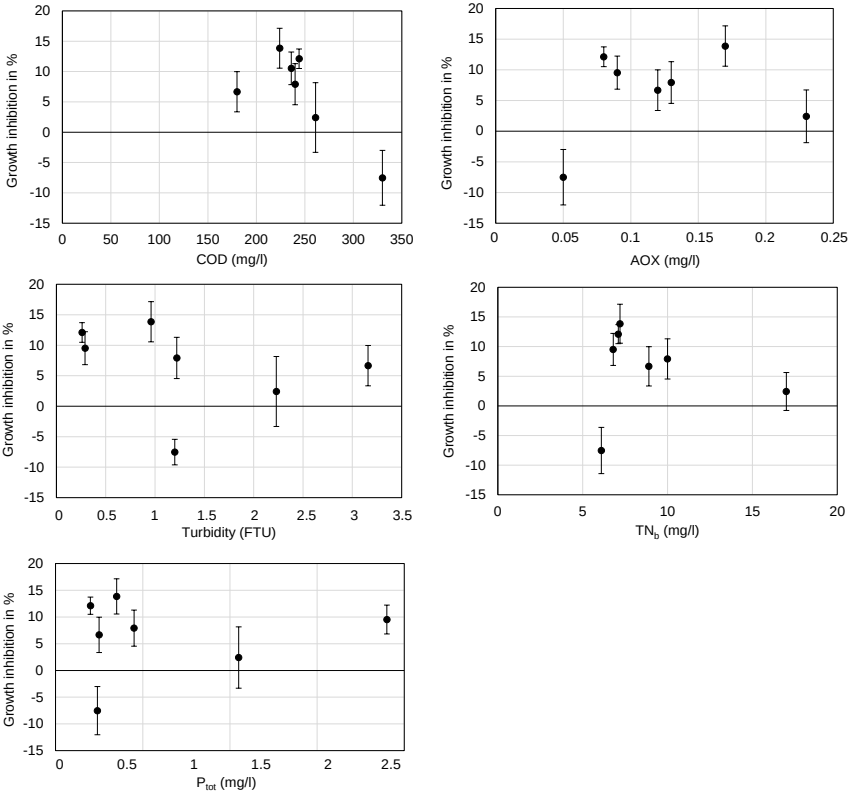


Figure 37: Correlation between measured parameters (COD, AOX, turbidity, TN_b and P_{tot}) of clarified effluent samples and growth inhibition for seven paper mills listed in **Table 6**.

The reasonable evaluation of the influence of chemical and physical parameters on growth inhibition can only be achieved by the definition of model wastewater. Here, each individual parameter must remain constant in a predefined series of experiments and the remaining parameters must vary within an experimental matrix. In addition, the investigated parameters and their mixtures must be in a similar range to the real paper mill wastewater.

6.9 Repeatability of the test results

The repeatability of Lemna minor test results was investigated by repeating the experiment three times under standardized conditions (see **Table 10**) for clarified effluent samples of seven paper mills.

The obtained results (G_w values) for clarified effluent samples based on frond number and frond area are summarized in **Table 13**.

Table 13: Obtained G_w values for the investigated paper mills after three times repeating the test.

	G_w_A			G_w_N		
Paper mill code	T 1	T 2	T 3	T 1	T 2	T 3
A	1	2	1	1	1	1
B	1	2	1	1	1	1
C	4	4	1	1	2	1
D	1	1	1	1	1	1
E	1	1	1	1	1	1
F	1	1	8	1	1	8
G	2	1	1	1	1	1

In 2 out of 7 tested clarified effluent samples, G_w value of 1 was calculated after three times repeating the test based on both observation parameters. The third Lemna minor test for paper mill F was classified as invalid. Due to the presence of fungi in the test samples as well as in the control approaches, the validity criteria according to the DIN ISO 20079:2006 could not be fulfilled. There is a small variation in G_w values ($1 \leq G_w \leq 2$) based on frond area for effluent samples from paper mill A, B and G. However, this value remains by 1 for all three investigations based on frond number. Among investigated paper mills, the highest G_w value based on both observation parameters is calculated for paper mill C. This value ranges from 1 to 4 in terms of frond area and from 1 to 2 based on frond number. Since the measured wastewater parameters such as COD, AOX, etc. indicated almost the

same value for each testing time, the deviation of obtained G_w values cannot be explained by the measured chemical and physical parameters.

Obviously higher G_w values in the range of $1 \leq Gw \leq 4$ based on frond area for samples treated with an anaerobic-aerobic process compared to aerobic-aerobic treated samples ($G_w = 1$) are recorded.

According to obtained results based on both observation parameters, higher G_w values are calculated based on the frond area. This can be related to the fact that some substances can influence the frond area more than the frond number, so evaluation of the test results only based on the frond number is not reliable [104]. This could also be observed by image analysis, where the number of fronds in test samples was comparable to those in control approaches but smaller in size.

As it can be seen from **Table 13**, the limit value of 10 % for growth inhibition is achieved mainly by D1 or D2 based on both observation parameters. However, the exact calculated value for growth inhibition and deviation of the results within three times test repetition for each test sample can not be conducted from the above table. To obtain a better idea of the variation in results for individual paper mills, as well as a comparison of resulting growth inhibition for different paper mills, the data are illustrated in the box and whisker plot based on frond number and frond area.

As indicated in **Figure 38** for clarified effluents from paper mills A and G with $1 \leq Gw \leq 2$ based on frond area (see **Table 13**), the median and upper quartile are under 10 %. This is evidenced by the fact, that more than 75 % of investigated samples showed growth inhibition of less than 10 % within three times repeating the test. The higher growth inhibitions were obtained for paper mill B in all repetitions compared to mill A and G with $1 \leq Gw \leq 2$. According to **Figure 38**, no data point with a value of higher than 10 % is recorded for paper mills D and E. The high standard deviation is also obtained in the negative range, which is related to a high growth rate of Lemna minor in these samples. For paper mill F, all measured points were in the same range and almost under 10 %. Due to the calculated median (6.45 %) and negligible standard deviation in the positive range, the clarified effluent of paper mill F could prove the repeatable results for growth inhibition of less than 10 % within three times repeating the test. The highest growth inhibition values are obtained for paper mill C, which is significantly higher than 10 %. For better comparison, the obtained results of dilution level 2 for paper mills with $G_w > 1$

are presented in **Figure 39**. The resulting growth inhibitions based on the frond area for D2 indicated no data point above 10 % for paper mills A, B, and G. It can be proven by the Lemna minor test under standardized conditions (see **Table 10**) the repeatable results in the range of 1 ≤ Gw ≤ 2 based on frond area for six out of seven investigated paper mills can be ensured.

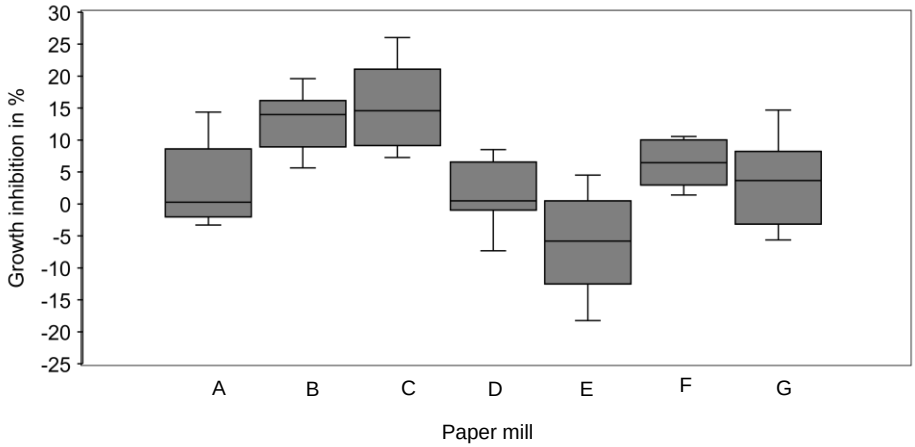


Figure 38: Calculated growth inhibitions (based on frond area) for D1 of clarified effluent samples within three times repeating the test (total sample size of 9 for each paper mill).

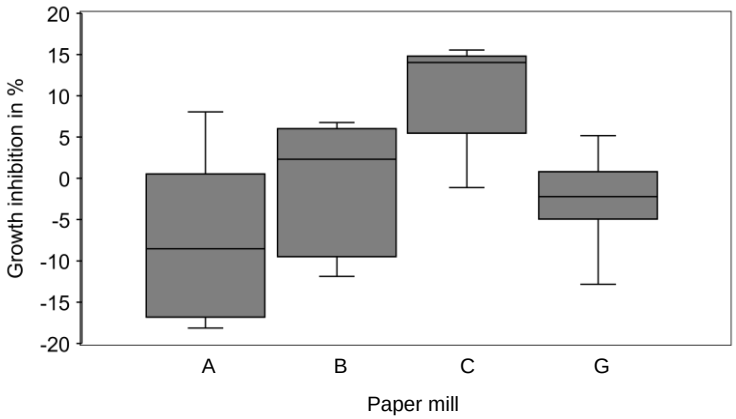


Figure 39: Calculated growth inhibitions (based on frond area) for D2 of clarified effluent samples within three times repeating the test (total sample size of 9 for each paper mill).

Similar to the above graphs, the calculated growth inhibitions based on frond number are demonstrated in **Figure 40** for D1.

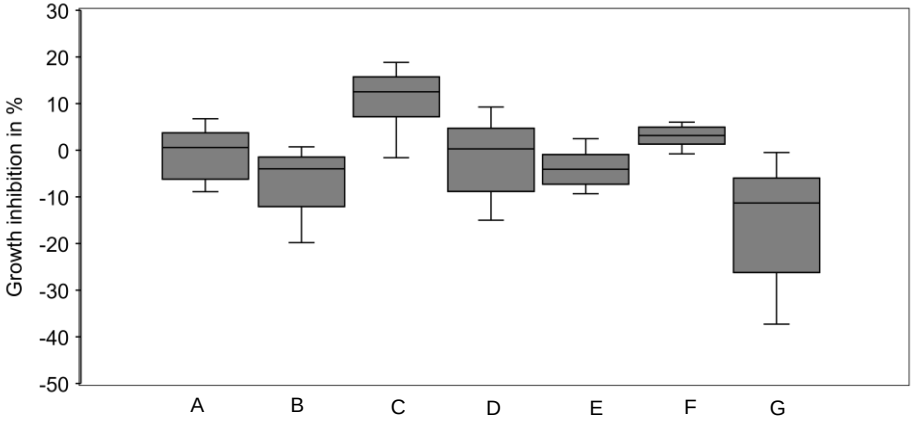


Figure 40: Calculated growth inhibitions (based on frond number) for D1 of clarified effluent samples within three times repeating the test (total sample size of 9 for each paper mill).

In contrast to illustrated results in **Figure 38** based on frond area, the obtained growth inhibitions regarding frond number for all investigated samples excluding effluents of mill C are below 10 %. For almost all paper mills, the higher standard deviation is obtained in the negative range, which relates to no critical toxic effect of samples on Lemna minor. Moreover, the deviation between calculated growth inhibition in each sampling time is considerably smaller compared to the results based on the frond area.

To summarize, in six of seven investigated paper mills the repeatable G_w values between 1 and 2 are obtained based on the two observation parameters. According to the box and whisker plot, calculated growth inhibitions after three times repeating the test are almost below 10 %. In most cases, the greater standard deviation is obtained in the negative range, which can be considered uncritical for test evaluation.

6.10 Reduction of the Lemna minor test duration

The calculated growth inhibitions for undiluted clarified effluent samples (D1) of seven paper mills within seven days of the experiment are demonstrated in **Figure 41** and **Figure 42** based on frond number and frond area, respectively. The remarkable change in growth inhibition from day 2 to day 5 can be observed in both figures. Growth inhibition of Lemna minor changed only slightly between day 5 and 7. Moreover, the same G_w value after five and seven days was obtained for all investigated clarified effluent samples.

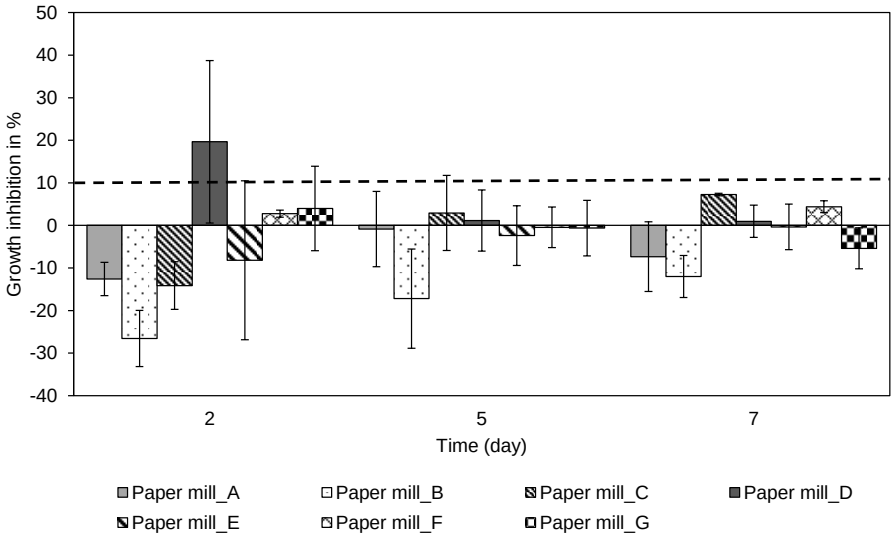


Figure 41: Growth inhibition (based on frond number) for clarified effluent samples (D1) from seven paper mills within seven days.

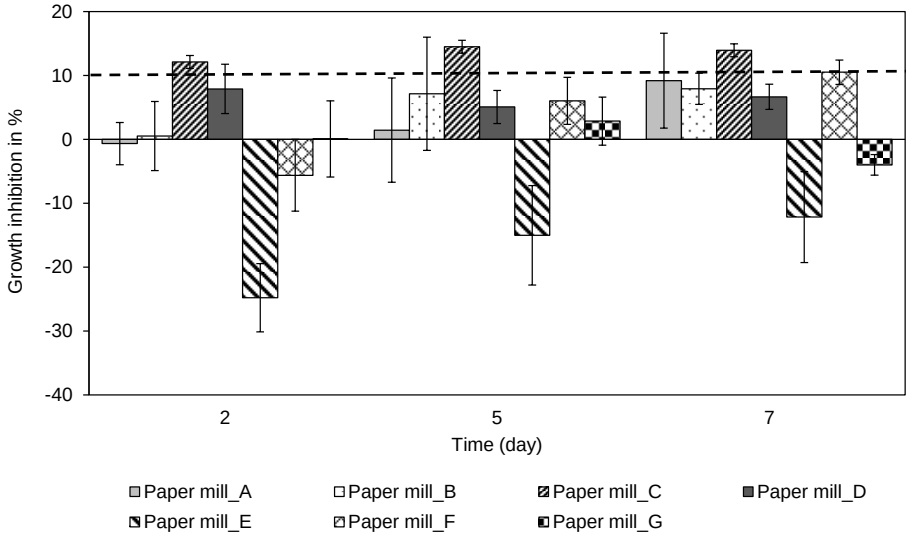


Figure 42: Growth inhibition (based on frond area) for clarified effluent samples (D1) from seven paper mills within seven days.

According to the previous results of this work (see **Table 13**), for almost all investigated paper mills a growth inhibition of lower than 10 % was obtained by D1 or D2. This supports the fact that substances contained in clarified effluent of paper mills have no critical toxic effect on Lemna minor. To compare the growth rate of Lemna minor after five and seven days, the measured data for control and reference approaches as well as for four different dilution levels are shown in **Figure 43**.

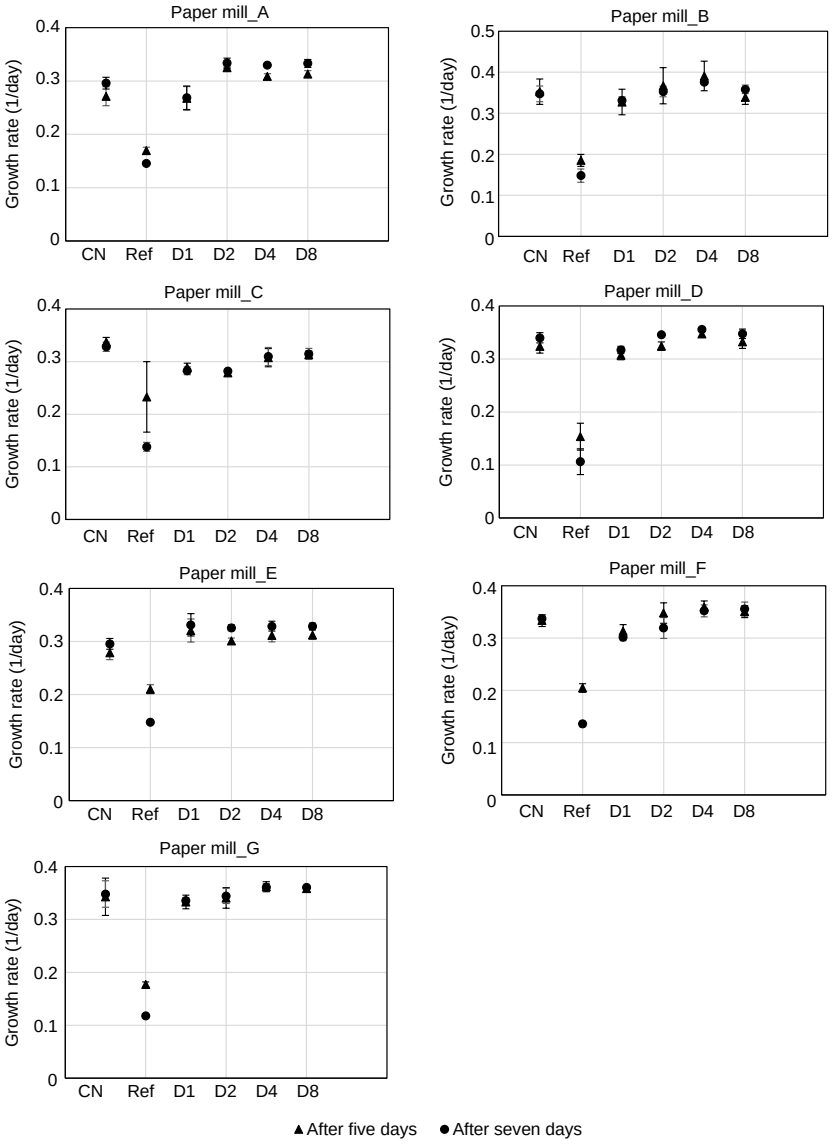


Figure 43: The growth rate of Lemna minor (based on frond area) in the dilution levels tested, including control and reference approaches, after five and seven days.

According to **Figure 43**, the growth rate of investigated samples indicates almost the same value after five and seven days. These are comparable and, in some cases, even greater than the growth rate of Lemna minor in control approaches. The major difference between the growth rate of control and dilution levels (D1 and D2) was obtained for paper mill C, which also indicated the highest G_w value among the tested paper mills (see **Table 13**). Even for a clarified effluent sample of Paper mill C with the most negative effect on Lemna minor, the growth rate after five and seven days is very similar. **Figure 43** indicates a substantial decrease in the growth rate of Lemna minor in the reference approaches from day 5 to day 7. This can also prove the fact that the presence of toxic substances in investigated sample negatively influences the growth rate of Lemna minor over time. If so, both the growth inhibition and the G_w value cannot be the same after five and seven days. Therefore, a defined time is required for accurate toxicity evaluation of the investigated sample.

According to the obtained results, clarified effluents of paper mills have only a negligible negative effect on Lemna minor growth rate. This effect can also be observed in the first five days of the Lemna minor test. The growth rate, as well as growth inhibition, remain almost constant after five days. Furthermore, there is no noticeable change or anomalies in Lemna minor between the fifth and seventh day of the experiment observed within this study.

7 Summary and outlook

The main objective of this work was to investigate the main parameters influencing the result of the Lemna minor test. This was conducted by testing clarified effluent of several different paper mills. The selection of paper mills was based on previous investigations. Hence, clarified effluent samples from paper mills using anaerobic-aerobic wastewater treatment were identified as the samples with the highest G_w value, followed by clarified effluents from mills with a deinking plant. In the context of research describing the standardized Lemna minor test procedure, a significant correlation was identified between sample preparation as well as conservation conditions and the test results. In the majority of sampling scenarios, samples cannot be analyzed immediately after sampling, thus it is necessary to determine the conservation conditions causing the lowest modification of growth inhibition, as well as the G_w value. Here, three different conservation temperatures including room temperature (24°C), refrigerator (4°C), and freezing (-18°C) was tested over four weeks. The smallest change in growth inhibition was obtained for samples kept frozen for up to two weeks. The next step was to examine the change in TSS value over time after freezing. A significant increase in TSS value after conservation under frozen conditions was observed for all investigated samples. Hence, four different methods for separating colloidal and solids from clarified effluent samples have been investigated over time. It could be observed that filtration of samples via black ribbon filter paper resulted in the lowest change in growth inhibition over time.

The influence of pH value on the Lemna minor test result was investigated first by using the filtrate of the disintegrated paper sample. Thus, a sample with the same properties (chemical and physical parameters) should be used for screening different pH values. Among tested pH values ranging from 5.0 to 8.0, samples with adjusted pH of 5.5 and 6.0 could show the lowest growth inhibition. In this study, a significant change in the pH of samples was observed over seven days of the experiment. However, the purpose was to determine the pH range, which provides the best conditions for Lemna minor growth. Subsequently, the clarified effluent of paper mill A (with anaerobic-aerobic treatment) was tested with the adjusted pH of 5.5 and 6.0 over a conservation time of up to four weeks. Here, a pH of 5.5 resulted in the lowest change of growth inhibition over time.

To define reliable dilution level ranges for testing clarified effluent of paper mills using Lemna minor test, the NOEC of the tested samples was calculated using ANOVA and Dunnett's test. In three of six clarified effluent samples, the NOEC was determined to be D8, and in three other samples, the NOEC was determined to be D4 or D2 based on two observation parameters. Thus, from D8 no toxic effect was observed on Lemna minor, the investigation of clarified effluent samples can be carried out with four dilution levels (D1, D2, D4, D8).

Within the multiple screening in paper mills, the stepwise reduction of growth inhibition along the wastewater treatment process was observed for all investigated paper mills. The most remarkable aspect was that wastewater samples from paper mills producing comparable paper types and using identical wastewater treatment techniques indicated different growth inhibitions at the same treatment stages. The resulting G_w value of untreated wastewater samples from graphic paper mills showed a significantly lower value ($8 \leq G_w \leq 12$) compared to primary influent samples of corrugated board and hygiene paper manufacturers ($G_w \geq 24$). From the studied graphic paper mills, mill G with anaerobic-aerobic wastewater treatment technique indicated the highest G_w value for both the primary effluent and the clarified effluent samples. For all investigated paper mills, almost the same level of growth inhibition was reported for the secondary effluent and clarified effluent samples.

For investigated clarified effluents from seven paper mills, there were no significant correlations between the growth inhibition of Lemna minor and the investigated parameters COD, AOX, turbidity, surface tension, nitrogen, and phosphorus content.

Samples of primary influent and primary effluent revealed higher concentrations of COD, AOX, and turbidity than those of secondary effluent and clarified effluent. These higher levels of wastewater contaminations are associated with higher growth inhibition. However, if an interaction of several factors or a direct effect of one of these factors is responsible for a higher growth inhibition cannot be determined from this investigation. Moreover, the expected positive effect of a higher concentration of nitrogen and phosphorus compounds in primary influent and effluent samples on Lemna minor growth rate was superimposed by other effects and could not influence growth inhibition.

The valid assessment for the influence of the wastewater parameters on test results could not be fulfilled in the scope of this work due to testing the real wastewater sample. Defining a model wastewater is the only option to estimate the influence of chemical and physical parameters on growth inhibition. However, it remains uncertain whether the exact determination of individual wastewater parameters and their interactions and effect on Lemna minor growth inhibition is an urgent topic for paper mills, as the wastewater parameters for clarified effluents from paper mills identified in this work do not exceed the values listed in annex 28. Furthermore, the resulting growth inhibitions for clarified effluent are almost associated with those of harmless samples for the environment. Of course, the primary influent of paper mills indicates a high level of contamination, but this can be treated well using the current wastewater treatment techniques.

In two out of seven tested clarified effluent samples, a G_w value of 1 was calculated for all three sampling times based on both observation parameters (frond number and frond area). There was a small variation in G_w values ($1 \leq G_w \leq 2$) based on frond area for effluent samples from paper mills A, B, and G. However, this value remained at 1 for all three investigations based on the frond number. Among investigated paper mills, the highest G_w value based on both observation parameters was calculated for paper mill C. This value ranged between 1 and 4 regarding the frond area and between 1 and 2 based on the frond number. A significantly larger G_w value in the range of $1 \leq Gw \leq 4$ based on frond area was observed for samples treated with an anaerobic-aerobic process compared to aerobic-aerobic treated samples ($G_w \leq 2$). According to the box and whisker plot for resulting growth inhibitions after three times repeating the test, for paper mills with $1 \leq G_w \leq 2$ (based on frond area) median, as well as upper quartile, was obviously under 10 % for D1. These paper mills had no data point higher than 10 % for D2 based on the frond area. The obtained growth inhibitions regarding frond number for all investigated samples excluding clarified effluents of mill C were under 10 %. Moreover, all investigated paper mills indicated a higher standard deviation in the negative range, which relates to no critical toxic effect of samples on Lemna minor. Remarkably, the deviation between calculated growth inhibitions based on frond number in each sampling time was lower compared to the results based on frond area.

The increase in growth inhibition from day 2 to day 5 could be observed for all conducted tests. Whereas only a negligible change in growth inhibition of Lemna minor occurred from day 5 to day 7. For all examined clarified effluent samples, the

G_w value was the same after five and seven days. The obtained results in this study indicate that clarified effluents of paper mills do not adversely affect Lemna minor growth rates. This effect is also apparent in the first five days of the test. The growth rate, as well as growth inhibition, remains almost constant after five days. Lemna minor does not seem to exhibit any noticeable changes between the fifth and seventh days of the experiment within this study.

Within the scope of this work, the main parameters causing the variation of test results were defined. Consequently, the Lemna minor test according to DIN EN ISO 20079 was further developed with a focus on the investigation of paper mills effluent. The sensitivity of the test to water contamination was demonstrated by studying the wastewater at multiple stages of treatment. Moreover, the repeatability of the test results based on the developed test procedure could be proven in the PMV laboratory for eight paper mills. However, before the recommendation of the test as a toxicity assessment in the paper industry, the reproducibility of the test results has to be further investigated by testing the same sample in different laboratories under defined conditions in this work.

The image analysis program is the most crucial factor, which can potentially cause the deviation in test evaluation within different laboratories. This can be more critical in the case of plant overlapping for evaluation based on frond area. It is recommended to use the same program with the same settings for the evaluation of samples tested in different laboratories.

Based on the obtained results, different growth inhibition could be identified for investigated paper mills. This varies from negative value to higher than 10 %. The substances or maybe the interaction of some substances, which are responsible for different values of growth inhibition, can be the topic of future studies.

Since the FET has been often used as a toxicity test for paper mills effluent, finding the correlation between Lemna minor test and FET and comparing their sensitivity to toxicants is reasonable before the inclusion of any toxicity test in Annex 28.

List of abbreviations

AbwV	Wastewater Ordinance (Abwasserverordnung)
AFT	Acute fish test
AOX	Adsorbable organic halogens
BMU	Federal Ministry for the Environment, Nature Conservation and Nuclear Safety
BOD_5	Biochemical oxygen demand
CN	Control sample
CO	Company
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
D(x)	Dilution level (x = ratio)
DBP	Dibutyl phthalate
DEP	Diethyl phthalate
DIBP	Diisobutyl phthalate
EC_x	Concentration of a test sample for which a growth inhibition of x % was determined compared to the control.
EDC	Endocrine-disrupting Chemicals
EGSB	Expanded granular sludge bed
FeS	Iron (II) sulfide
FET	Fish embryos toxicity test
G_{Ei}	Maximum concentrated dilution at which a critical growth inhibition is not exceeded. It is used in the fish embryos test.
G_w(_X)	Maximum concentrated dilution at which a critical growth inhibition is not exceeded. It is used in Lemna

	minor test (based on observation parameter X, with X = frond number N or frond area A).
H_2S	Hydrogen sulfide
IC	Internal circulation
LC 50	Lethal concentration. The concentration of a toxic substance dissolved in water, which can kill 50 % of a test population
NOEC	Lowest dilution level without toxic effect (No Observed Effect Concentration)
N_{tot}	Total nitrogen
OECD	Organization for Economic Co-operation and Development
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyls
PMV	Institute of Paper Technology and Mechanical Process Engineering, Technical University of Darmstadt
P_{tot}	Total phosphorus
PTS	Paper Technology Fundation (Papiertechnische Stiftung)
REACH	Regulation, Evaluation, Authorization and Restriction of Chemicals
Ref	Reference sample
T	Test repetition
T_{effl}	Temperatur of clarified effluent
TN_b	Total nitrogen bound
TOC	Total organic carbon
TSS	Total suspended solids

UASB	Upflow anaerobic sludge blanket
UBA	Federal Environmental Agency (Umweltbundesamt)
VDP	Association of German Paper Manufacturing (Verband Deutscher Papierfabriken e. V.)
WFD	Water Framework Directive
WWTP	Wastewater treatment plant

List of symbols

d	Critical value of Dunnett's test
$i_r(x)$	Percentage inhibition of the specific growth rate r of the observation parameter X (X = frond number N or frond area A)
E_i	Referred to fish embryos test
G	Maximum Concentrated dilution at which a critical growth inhibition is not exceed
MSD	Minimum significant difference for Dunnett's test
r	Growth rate per day
R^2	Coefficient of determination
r_c	Mean specific growth rate of the control
r_t	Mean specific growth rate of the test concentrations
t_i	Difference between the mean of each concentration and the mean of control samples (for Dunnett's test)
t_z	Experiment day
w	Referred to Lemna minor test
x	Values of the observation parameter: frond number or frond area
x_t	Values of the observation parameter after t days
x_{tz}	Values of the observation parameter after t_z days (z: number of days)
y	Regression line

Appendices

I. Calculation of NOEC

For estimation of NOEC, the ANOVA test is used to calculate the mean specific growth rate and the residual standard deviation over the replicates for each test concentration. The resulting mean for each test concentration is then compared to the control mean using Dunnett's test as a suitable multiple comparison method. The t_i value of each dilution level can be calculated using the following formula:

$$t_i = Y_i - Y_1 \tag{3}$$

To compare the calculated mean for each concentrations group with the mean of control group, the Minimum Significant Difference (MSD) is calculated and used according to equation (4):

$$MSD = d \cdot \sqrt{\frac{2MS_w}{n}} \tag{4}$$

t_i t statistics of each concentration

MSD Minimum Significant Difference

Y_1 Mean of control

Y_i Mean of concentration i

MS_w The Mean Squares of the "Within Group" from ANOVA test

n Number of replicates

d Critical value of Dunnett's test (The value found in Dunnett's table for a given alpha level, number of groups, and group sample sizes) [106].

The lowest dilution level (corresponding to the highest effluent concentration) that can prove $t_i > MSD$ is defined as NOEC. Thus, the growth of Lemna minor in these group samples is equal to or better than those in the control group and no toxic effect of a sample on the test organism can be observed.

In this work, NOEC was calculated for all samples, which were stored under frozen conditions for up to two weeks and filtered via black ribbon filter paper (a standardized condition described in chapter **6.6**). This is based on the raw data of the growth rate based on the frond area. The reason is that the frond area is a more sensitive observation parameter compared to the frond number. Furthermore, the obtained results based on the frond area for all investigated samples were associated with a lower growth rate (higher growth inhibition). Therefore, the resulting NOEC based on the frond area definitely complies with the results of the frond number, too. The calculated NOEC for each paper mill are summarized in the following tables.

Paper mill A

The clarified effluent sample is filtered via black ribbon filter paper and stored under frozen conditions.

A) Test execution one day after sampling.

Table A1: Growth rate of Lemna minor for different dilution levels. The test is performed one day after sampling.

Sample	Control	D1	D2	D4	D8	D12	D24
Growth rate 1/day (based on frond area)	0.283	0.298	0.345	0.331	0.342	0.336	0.326
	0.293	0.245	0.333	0.330	0.332	0.346	0.323
	0.290	0.262	0.322	0.326	0.324	0.337	0.338

Table A2: Results of ANOVA test.

α =0.05	Between groups	Within groups
Sum of squares (SS)	0.013	0.002
Degree of freedom	6	14
Mean square	0.0022	0.0001
F	14.77	
P value	2.45E-05	
F critical	2.84	

Table A3: Results of Dunnett's test.

Sample	Mean value	t_i	$t_i > MSD$(=0.029)
Control	0.289		
D1	0.268	-0.02	No
D2	0.333	0.044	Yes = NOEC
D4	0.329	0.040	Yes
D8	0.332	0.043	Yes
D12	0.34	0.051	Yes
D24	0.329	0.04	Yes

B) Test execution two weeks after sampling.

Table A4: Growth rate of Lemna minor for different dilution levels. The test is performed two weeks after sampling.

Sample	Control	D1	D2	D4	D8	D12	D24
Growth rate 1/day (based on frond area)	0.294	0.283	0.356	0.374	0.326	0.329	0.333
	0.306	0.285	0.326	0.309	0.324	0.336	0.352
	0.294	0.290	0.336	0.365	0.361	0.343	0.336

Table A5: Results of ANOVA test.

α =0.05	Between groups	Within groups
Sum of squares (SS)	0.010	0.004
Degree of freedom	6	14
Mean square	0.001	0.0003
F	5.83	
P value	0.003	
F critical	2.84	

Table A6: Results of Dunnett's test.

Sample	Mean value	t_i	$t_i > MSD$(=0.041)
Control	0.298		
D1	0.286	-0.012	No
D2	0.340	0.042	Yes
D4	0.349	0.051	Yes
D8	0.337	0.038	No
D12	0.336	0.037	No
D24	0.340	0.042	Yes

In this investigation, the NOEC is first obtained by D2. Thereafter, the growth of Lemna minor deteriorated by D8 and D12. Since the growth inhibition of these samples was still below 10 %, the samples were classified as harmless. However, the determination of NOEC is not possible in this case.

Paper mill E

The clarified effluent sample is filtered via black ribbon paper filter and sored under frozen conditions.

A) Test execution one day after sampling.

Table A7: Growth rate of Lemna minor for different dilution levels. The test is performed one day after sampling.

Sample	Con-	D1	D2	D4	D8	D12	D24
Growth rate 1/day (based on frond area)	0.281	0.343	0.318	0.338	0.337	0.338	0.331
	0.299	0.349	0.330	0.331	0.336	0.375	0.344
	0.306	0.301	0.329	0.326	0.332	0.347	0.327

Table A8: Results of ANOVA test.

α =0.05	Between groups	Within groups
Sum of squares (SS)	0.005	0.002
Degree of freedom	6	14
Mean square	0.0009	0.0001
F	4.69	
P value	0.008	
F critical	2.84	

Table A9: Results of Dunnett's test

sample	Mean value	t_i	$t_i > MSD$(=0.033)
Control	0.295		
D1	0.331	0.035	Yes
D2	0.325	0.030	No
D4	0.332	0.036	Yes = NOEC
D8	0.335	0.040	Yes
D12	0.354	0.058	Yes
D24	0.334	0.038	Yes

B) Test execution two weeks after sampling

Table A10: Growth rate of Lemna minor for different dilution levels. The test is performed two weeks after sampling.

Sample	Con-	D1	D2	D4	D8	D12	D24
Growth rate 1/day (based on frond area)	0.321	0.391	0.333	0.352	0.365	0.361	0.360
	0.315	0.364	0.352	0.346	0.376	0.388	0.372
	0.317	0.313	0.370	0.356	0.381	0.363	0.374

Table A11: Results of ANOVA test.

α =0.05	Between groups	Within groups
Sum of squares (SS)	0.006	0.004
Degree of freedom	6	14
Mean square	0.001	0.0003
F	3.33	
P value	0.029	
F critical	2.84	

Table A12: Results of Dunnett's test.

Sample	Mean value	t_i	$t_i > MSD$(=0.043)
Controle	0.318		
D1	0.356	0.038	No
D2	0.352	0.034	No
D4	0.352	0.033	No
D8	0.374	0.056	Yes = NOEC
D12	0.371	0.053	Yes
D24	0.369	0.051	Yes

Paper mill H

The clarified effluent sample is filtered via black ribbon filter paper and stored under frozen conditions.

A) Test execution one day after sampling.

Table A13: Growth rate of Lemna minor for different dilution levels. The test is performed one day after sampling.

Sample	Con-	D1	D2	D4	D8	D12	D24
Growth rate 1/day (based on frond area)	0.333	0.342	0.343	0.366	0.369	0.364	0.349
	0.339	0.355	0.343	0.345	0.352	0.358	0.377
	0.336	0.354	0.343	0.333	0.357	0.361	0.366

Table A14: Results of ANOVA test.

α =0.05	Between groups	Within groups
Sum of squares (SS)	0.001	0.001
Degree of freedom	6	14
Mean square	0.0003	8.86E-05
F	3.39	
P value	0.028	
F critical	2.84	

Table A15: Results of Dunnett's test.

Sample	Mean value	t_i	$t_i > MSD$(=0.022)
Control	0.336		
D1	0.350	0.014	No
D2	0.344	0.008	No
D4	0.348	0.012	No
D8	0.359	0.023	Yes = NOEC
D12	0.361	0.024	Yes
D24	0.364	0.027	Yes

B) Test execution two weeks after sampling.

Table A16: Growth rate of Lemna minor for different dilution levels. The test is performed two weeks after sampling.

sample	Con-	D1	D2	D4	D8	D12	D24
Growth rate 1/day (based on frond area)	0.376	0.383	0.378	0.403	0.407	0.414	0.416
	0.379	0.395	0.399	0.413	0.419	0.408	0.413
	0.382	0.439	0.380	0.406	0.416	0.408	0.405

Table A17: Results of ANOVA test.

α =0.05	Between groups	Within groups
Sum of squares (SS)	0.003	0.002
Degree of freedom	6	14
Mean square	0.0005	0.0001
F	3.61	
P value	0.022	
F critical	2.84	

Table A18: Results of Dunnett's test.

Sample	Mean value	t_i	$t_i > MSD$(=0.029)
Kontrolle	0.379		
D1	0.406	0.026	No
D2	0.386	0.006	No
D4	0.407	0.028	No
D8	0.414	0.034	Yes = NOEC
D12	0.410	0.030	Yes
D24	0.412	0.032	Yes

II. Paper mills questionnaire

Paper mill (Mill code)	
Produced paper type	
Production capacity (ton per year)	
Wastewater per ton of produced paper (m^3/t)	
Fresh water quantity (m^3/t)	
Wastewater treatment technique	
Wastewater parameter (clarified effluent)	
COD homog. (mg/l)	
P_{tot} (mg/l)	
NO_2-N (mg/l)	
NO_3-N (mg/l)	
NH_4-N (mg/l)	
TSS (mg/l)	
Turbidity (FTU)	
AOX (mg/l)	
TN_b (mg/l)	
Temp. (°C)	
pH value	
Conductivity (mS/cm)	
Addition of chemical additives	
Production (e.g. retention agent fixing agent, complexing agent)	
WWTP (e.g. defoamer, flocculant)	

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Aziziyanesfahani P., Krebs T., Kersten A., Schabel S. (2021):

"Lemna minor test tool for toxicity assessment of paper mill effluent."

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[Artikel]

Aziziyanesfahani P., Kersten A., Schabel S. (2021):

„Wasserlinsen-Test. Untersuchung der Eignung als Überwachungsparameter für Papierfabriksabwässer."

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[Artikel]

Aziziyanesfahani P., Kersten A., Schabel S. (2021):

„Untersuchung zur Eignung des Wasserlinsen-Tests als Überwachungsparameter in der Matrix Papierfabriksabwasser."

Darmstadt, AiF 19761, DOI: 10.26083/tuprints-00018635

[Report]

List of supervised student work

Colin Wawrik (2021)**:**

„Untersuchung der Eignung des Wasserlinsen-Wachstumshemmtests als Überwachungsparameter für Papierfabriksabwässer."

[Bachelor Thesis]