Application of in vitro bioassays to monitor pharmaceuticals in water: A synthesis of chronological analysis, mode of action, and practical insights

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Application of *in vitro* bioassays to monitor pharmaceuticals in water: A synthesis of chronological analysis, mode of action, and practical insights

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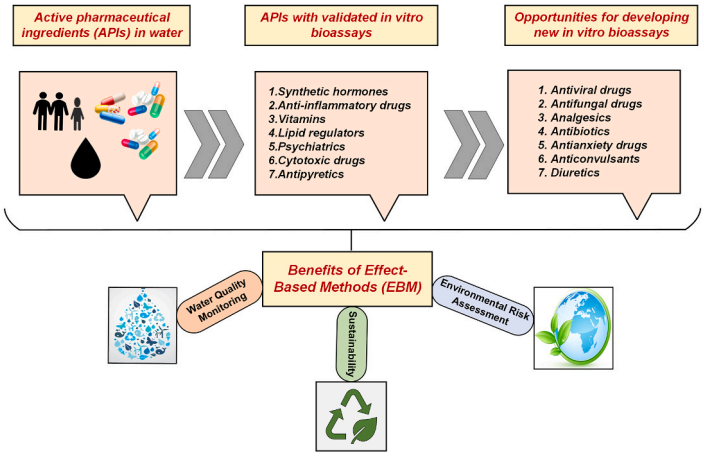
HIGHLIGHTS

- *In vitro* bioassays can assess the toxicity of pharmaceuticals in water.
- Pharmaceuticals classified based on their mode of action.
- Current *in vitro* bioassay coverage of pharmaceutical classes explored.
- About 8 out of 34 pharmaceutical classes possess validated *in vitro* bioassays.
- Opportunities to develop novel bioassays for water quality monitoring highlighted.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutical compounds in wastewater have emerged as a significant concern for the aquatic environment. The use of *in vitro* bioassays represents a sustainable and cost-effective approach for assessing the potential toxicological risks of these biologically active compounds in wastewater and aligns with ethical considerations in research. It facilitates high-throughput analysis, captures mixture effects, integrates impacts of both known and unknown chemicals, and reduces reliance on animal testing. The core aim of the current review was to explore the practical application of *in vitro* bioassays in evaluating the environmental impacts of pharmaceuticals in wastewater. This comprehensive review strives to achieve several key objectives. First, it provides a summary categorisation of pharmaceuticals based on their mode of action, providing a structured framework for understanding their ecological significance. Second, a chronological analysis of pharmaceutical research aims to document their prevalence and trends over time, shedding light on evolving environmental challenges. Third, the review critically analyses existing bioassay applications in wastewater, while also examining bioassay coverage of representative compounds within major pharmaceutical classes. Finally, it explores the potential for developing innovative bioassays tailored for water quality monitoring of pharmaceuticals, paving the way for more

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robust environmental monitoring and risk assessment. Overall, adopting effect-based methods for pharmaceutical monitoring in water holds significant promise. It encompasses a broad spectrum of biological impacts, promotes standardized protocols, and supports a bioassay test battery approach indicative of different endpoints, thereby enhancing the effectiveness of environmental risk assessment.

List of abbreviations

API	Active Pharmaceutical Ingredient
AR	Androgen Receptor
ATP	Adenosine Triphosphate
BLT	Bacterial Luminescence Toxicity
CEC	Contaminant of Emerging Concern
COX	Cyclooxygenase
EBM	Effect-based Methods
EBT	Effect-Based Trigger Values
EDC	Endocrine Disrupting Compound
EE2	17α-Ethinylestradiol
ELISA	Enzyme Linked Immunosorbent Assay
ER	Estrogen Receptor
FAITH	Fast Assembly Inhibitor Test for HIV
FLISA	Fluorescence-Linked Immunosorbent Assay
GABA	Gamma-Aminobutyric Acid
GR	Glucocorticoid Receptor
HRMS	High Resolution Mass Spectrometry
LDL	Low-Density Lipoprotein
MAO	Monoamine Oxidase
MAT	Monoamine Transporter
MoA	Mode of Action
NSAID	Non-Steroidal Anti-Inflammatory Drug
NTA	Non-Target Analysis
PPAR	Peroxisome Proliferator-Activated Receptor
PR	Progesterone Receptor
PXR	Pregnane X Receptor
SNRI	Serotonin & Norepinephrine Reuptake Inhibition
SSRI	Selective Serotonin Reuptake Inhibition
TGF	Transforming Growth Factor
USEPA	United States Environmental Protection Agency
XETA	Xenopus Embryonic Thyroid Assay
YAS	Yeast Androgen Screen
YES	Yeast Estrogen Screen

1. Introduction

Pharmaceuticals are an important class of contaminant of emerging concern (CEC) in aquatic environments, and are ubiquitous in fresh and marine surface waters, drinking waters, and wastewater (Madikizela et al., 2017; Vumazonke et al., 2020). Many active pharmaceutical ingredients (APIs) pose toxicological risks to non-target organisms even at trace concentrations (Afonso-Olivares et al., 2013; Daniels et al., 2018). Globally, pharmaceuticals have an estimated consumption of 100,000 to 200,000 tons per annum (Dinh et al., 2011; Vumazonke et al., 2020). Between 5% and 90% of pharmaceuticals are excreted from the body in the form of the active ingredient, or parent compound, with the remainder excreted as metabolites, depending on their chemical characteristics (da Silva and Oliveira, 2018). These APIs are released in sewer systems and ultimately enter the environment via the discharge of effluents from wastewater treatment plants (Madikizela et al., 2017; Tanveer et al., 2022), or through the improper disposal of unfinished or unused drugs (Ayman and Işık, 2015; Wilkinson et al., 2022). Consequently, this causes contamination of natural water bodies, and has raised international concerns about the impacts of pharmaceutical pollution on water quality and ecosystem health (Fatta-Kassinos et al., 2011).

Pharmaceuticals are typically monitored by targeted chemical analysis (Kondor et al., 2020; Wang et al., 2022). One of the limitations of targeted chemical analysis is that, as the name implies, only "predetermined" or "known" chemicals are detected and measured. With thousands of pharmaceuticals in regular use, along with their diverse metabolites and environmental transformation products, it can be difficult to determine what compounds should be monitored (Escher et al., 2021). Non-target analysis (NTA) is an emerging analytical approach using high-resolution mass spectrometry (HRMS) to identify unknown or non-targeted chemicals present in a sample. However, while NTA can further expand the range of chemicals that can be detected, it does not provide information on the potential toxicity of identified chemicals, or their effect in mixtures. In addition, accurate quantification of chemical concentrations in NTA can be expensive and time-consuming. The application of *in vitro* bioassays and well plate-based *in vivo* assays, commonly referred to as effect-based methods (EBM), thus offers immense potential for enhancing water quality monitoring (Neale et al., 2020b, 2023). EBM can detect the effect of all chemicals in a sample that are active in the applied bioassay, including known and unknown chemicals. This accounts for mixture effects and is risk-scaled, with more potent chemicals having greater response in the assay compared to less potent compounds at similar concentrations (Escher et al., 2021). EBM can facilitate rapid and sensitive detection of chemical activity in biological systems at the cellular level (Connon et al., 2012). As a result, EBM have been proposed as a complementary approach to chemical analysis for a more holistic assessment of water quality. One of the limitations is that EBM alone has unable to identify individual chemicals that are contributing to the effects (Escher et al., 2021; Neale et al., 2023) and that's why EBM can combine with other techniques to provide more understanding of contamination and overcome the individual limitations. Further, by combining chemical analysis and EBM through iceberg modelling, it is possible to quantify the contribution of measured chemicals in a mixture or environmental sample to the observed effect (Neale et al., 2020a).

EBM have been applied to wastewater, recycled water, surface water and drinking water, to benchmark water quality (Escher et al., 2014; Leusch et al., 2018b), evaluate changes over time or after upgrading treatment processes (Cavallin et al., 2020), and to evaluate treatment efficiency (Leusch et al., 2014; Choi et al., 2023). Surface waters receiving wastewater effluent have previously been shown to induce effects in several *in vitro* assays (Müller et al., 2018), highlighting that wastewater treatment options are not completely effective at removing chemical pollutants and their associated biological effects (Melvin and Leusch, 2016). Assays indicative of different stages of cellular toxicity pathways, including induction of xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and cytotoxicity, have been applied for water quality monitoring. Further, some *in vitro* bioassays can provide an indication of possible chronic effects such as endocrine disruption, carcinogenicity and chronic stress (Escher et al., 2021). EBM present an attractive option for evaluating the potential toxic outcomes of APIs in wastewater based on their biological activity

(Desbiolles et al., 2018). EBM are already applied to detect some classes of APIs in water. For example, *in vitro* bioassays are commonly applied to detect trace concentrations of synthetic and natural hormones and other endocrine disrupting compounds (EDCs) in water (Leusch et al., 2017).

The aim of the current study was to explore the broad application of EBM for assessing biological effects induced by pharmaceuticals in wastewater. The main objectives of this review were to 1) summarize pharmaceutical classification based on mode of action (MoA), 2) chronologically analyse pharmaceutical research in wastewater to track substance prevalence and trends over time, 3) review available bioassay application in wastewater based on MoA, with representative chemicals in each pharmaceutical class also reviewed to examine the biological effects on organisms and the environment, and 4) discuss knowledge gaps and explore potential opportunities for developing new bioassays for water quality monitoring. Overall, the application of EBM for detecting pharmaceuticals could provide significant value by encompassing a wide array of biological impacts, progressing standardized protocols, and enabling comprehensive bioassay test batteries to look at multiple endpoints, thereby enhancing the effectiveness of environmental risk assessment.

2. Pharmaceuticals classification and literature search

There are many different ways to classify an API, including by chemical structure, by its principal pharmacological effect, by its physiological effect, by its cellular mechanism of action, or by its medical and diagnostic use (Page and Maddison, 2008). Starting from the US Food and Drug Administration list (FDA, 2015), we have classified APIs into 34 classes based on their mode of action (Table S1). A literature search of these pharmaceutical classes was undertaken through a comprehensive search of the Scopus database. Each API class descriptor was combined using the Boolean operator "AND" with the keyword "wastewater*" within the search string. The Scopus search was conducted in August 2023 on all literature published until that date. To limit the search outputs, review articles, editorials, conference papers, and articles written in languages other than English were excluded.

3. Annual consumption of active pharmaceuticals

The global consumption and prescription of substantial amounts of pharmaceuticals, encompassing a wide range of pharmaceutical classes used in human and veterinary medicines, has previously been documented (Boxall et al., 2014; Bouzas-Monroy et al., 2022). Estimates regarding pharmaceutical consumption are readily accessible due to their widespread availability (Fernandes et al., 2021). Table S2 provides a summary of consumption statistics for APIs commonly detected in water, sorted into 34 pharmaceutical classes, with their consumption quantified in kg per year (Whitacre et al., 2010; Boxall et al., 2012). These data have been extracted from research studies investigating the occurrence and fate of APIs present in the environment (Whitacre et al., 2010; Bouzas-Monroy et al., 2022). The estimation of API consumption data is based on prescription volumes, which may potentially undervalue the actual production levels and concentrations observed in the environment. This discrepancy is often due to patients retaining unused drugs in their households, typically because of changes in dosage or the discontinuation of medications. Further, unused pharmaceuticals are also regularly returned by the users to the pharmacies. This factor is frequently taken into account when calculating annual drug consumption, as these returns can make up a non-negligible percentage of the drugs initially distributed to consumers (Boxall, 2004; Boxall et al., 2014).

Antibiotics and hormones were the most highly manufactured pharmaceutical classes, with quantities reaching approximately 15.6 and 14.4 kilotons/year, respectively (Table S2). These, along with various anti-inflammatory medications, and antihistamines are produced in large volumes for humans, and also commonly used in poultry farming, aquaculture and livestock farming (Diáz-Cruz and Barceló, 2004; Boxall et al., 2012). Antibiotics are widely produced compared to other pharmaceutical classes and consumed worldwide to a great extent. Following antibiotics and hormones, anti-inflammatory drugs, such as ibuprofen, and analgesics, such as acetaminophen, are also sold in large amounts annually, with several hundred tons of anti-inflammatory and analgesics pharmaceuticals consumed each year in low-to-middle income countries (Ziylan and Ince, 2011). Considering their frequency and the potential for adverse toxicological effects, this represents a significant contribution to pharmaceutical pollution in both wastewater and surface water (Ahonen et al., 1991; Banik and Hossain, 2006). Various therapeutic categories including antiviral drugs, anticonvulsants, antidiabetic drugs, lipid regulators, psychiatric drugs, antipyretics, and anaesthetics are sold in quantities of less than 20,000 kg/year (Table S2).

3.1. Association between number of publications and annual production in wastewater

Globally, increasing research attention has been directed towards monitoring for APIs in wastewater. We explored whether any particular API classes had received more or less attention relative to their annual production. Fig. 1 highlights the relationship between the number of publications and annual production of pharmaceuticals in wastewater. This graphical representation highlights the relationship between the extent of published scientific research and the real-world prevalence of key classes of pharmaceuticals based on their production rates. Interestingly, the figure demonstrates a strong linear relationship between the number of studies and production volume, suggesting that research is appropriately driven by the potential risk posed by chemicals having high production volume in general. When considering pharmaceuticals ranked by production volume from highest to lowest, researchers have shown varying levels of interest. Eight pharmaceutical classes have received more attention than their production volume would indicate (falling more than 0.3 log units above the line of best fit): antibiotics, antiviral, anticonvulsant, antihypertensives, antidiabetics, lipid regulators, psychiatric and antipyretic compounds. In contrast, 8 pharmaceutical classes appear to have received less attention than their production volumes would warrant considering their consumption (falling more than 0.3 log units below the line of best fit): analgesics, vitamins, antidepressants, corticosteroids, anaesthetics, antacids, laxatives, and decongestants. The first four of these classes, with their moderate to high production rates, are likely the most crucial candidates for environmental monitoring and further study, as this relationship may suggest they are not receiving sufficient attention relative to their production and likely environmental occurrence.

Interestingly, the relationship illustrates how the scientific community's focus largely aligns with and responds to the pressing issue of adverse environmental effects caused by pharmaceuticals. Antibiotics have received considerable research attention since they are produced in very high volumes and widely prescribed. Many studies have identified significant environmental concerns with global antibiotics use, including the rise of antimicrobial resistance (Hiller et al., 2019). Other high volume production pharmaceuticals such as hormones and anti-inflammatory APIs also exhibit remarkable correlation between annual production and number of studies, again highlighting the research community's emphasis on ubiquitous pharmaceutical classes. Corticosteroids exhibited the most severe deviation from the average linear correlation (more than 0.6 log units below the line of best fit), suggesting that they may deserve more attention than they have received thus far. This visual representation provides an overview to illustrate where the scientific efforts should be concentrated within the specific category of pharmaceuticals, which can be valuable for readers seeking to identify research gaps (Fig. 1).

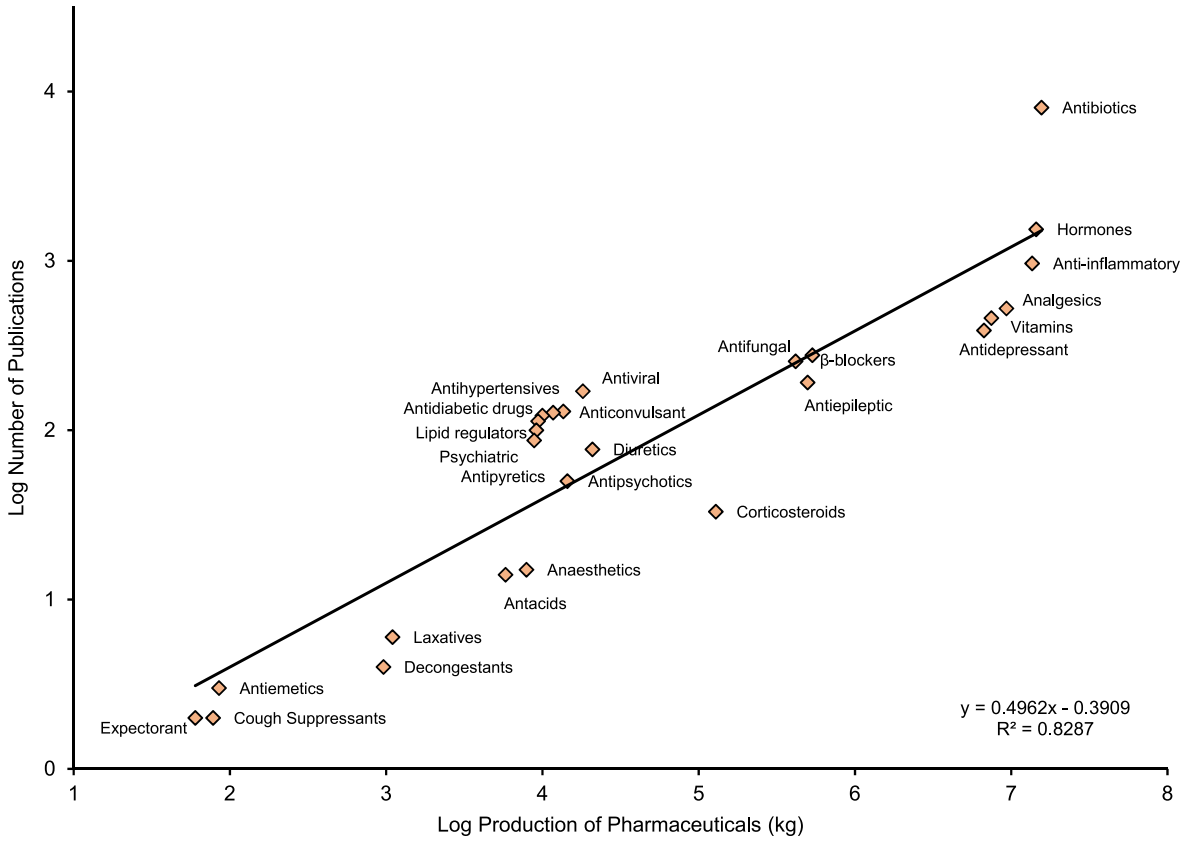

Fig. 1. Correlation between Publication Count and Annual Active Pharmaceuticals Production in Wastewater obtained by Linear Regression.

4. Evolution of research on pharmaceuticals in wastewater: A chronological analysis

Our literature search confirmed an increase in research focused on the occurrence of pharmaceuticals in wastewater that highlights their growing significance as environmental contaminants (Bengtsson-Palme et al., 2018). Antibiotics emerged as the category with the most extensive publication record and highest annual production, with their prominent status as an environmental concern and focus of monitoring highlighted by 8032 publications. Hormones and anti-inflammatory APIs similarly exhibited a considerable volume of research, with a total of 1535 and 965 publications, respectively, underscoring their comparatively higher research focus compared to other pharmaceutical classes. Slightly lower but still with a significant body of work, analgesics, vitamins, β-blockers, antidepressants, corticosteroids, antiepileptic, and antiviral drugs were investigated in wastewater by 523, 459, 277, 255, 215, and 191 studies, respectively (Fig. 2). Some pharmaceutical classes have received very minimal attention in wastewater. For example, despite considerable production volumes and medical usage, certain categories of pharmaceuticals, such as decongestants, antiemetics, expectorants, and cough suppressant medications, are underpinned by a lack of research, with fewer than 5 publications in our literature search dedicated to their exploration in wastewater. Antidiarrheal medications have been extensively used for the treatment of diarrheal infections. However, to our knowledge, there is currently just one study available in Scopus investigating the occurrence of antidiarrheal drugs in wastewater. Overall, this information helps identify a pathway for future study based on a chronological analysis of research attention framed against pharmaceutical production.

A chronological analysis was carried out for various pharmaceutical classes in water monitoring, to investigate research trends over time on a finer scale (Fig. S1). Antibiotics have received steadily increasing attention from the scientific community, particularly since the early 2000s, as illustrated in Figure S1 (I). The issue of antibiotic resistance has become a worldwide public concern, encompassing both environmental and health-related issues (Bengtsson-Palme et al., 2018; Mutuku et al., 2022). In response to this challenging situation, researchers have proactively addressed the problem of environmental contamination caused by antibiotics (Serra-Compte et al., 2021).

Hormones have likewise received significant and increasing attention, especially since the early 2000s (Figure S1,II), including 125 publications related to wastewater monitoring in 2022 alone. Purdom et al. (1994) investigated the estrogenic impacts leading to the presence of hermaphrodite fish in lagoons, ultimately attributing the toxicity to endocrine active compounds in sewage water. Using the vitellogenin protein as an indicator of estrogen stimulation in fish livers, the estrogenic activity was determined to be driven by natural and synthetic hormones such as 17β-estradiol and 17α-ethinylestradiol (EE2), an active pharmaceutical ingredient (API) in human contraceptive medicines, and alkylphenol polyethoxylates, which are generated through the biodegradation of detergents and surfactants in sewage water. Jobling et al. (1998) achieved a significant breakthrough when they revealed the phenomenon of intersexuality within wild roach populations. This condition had a profound impact on sexual growth and development, leading to a substantial body of work in the field of environmental monitoring of hormone-active compounds, including numerous APIs.

Research on anti-inflammatory drugs and analgesics increased noticeably in the mid-2000s (Figure S1, III-IV). Both groups of pharmaceuticals, which are regularly detected in wastewater (Guerra et al., 2014; Paíga et al., 2015), are utilized for reducing inflammation and relieving pain. Studies investigating vitamins in wastewater exhibited an interesting peak around the early 2010s, followed by a 4–5 year drop before picking up again sharply since 2018 (Figs. S1 and V). Vitamins such as A, D, E, K, B_6, B_{12} in wastewater are characterised by bio-resistant organic contaminants, inhibition of microbial activity and

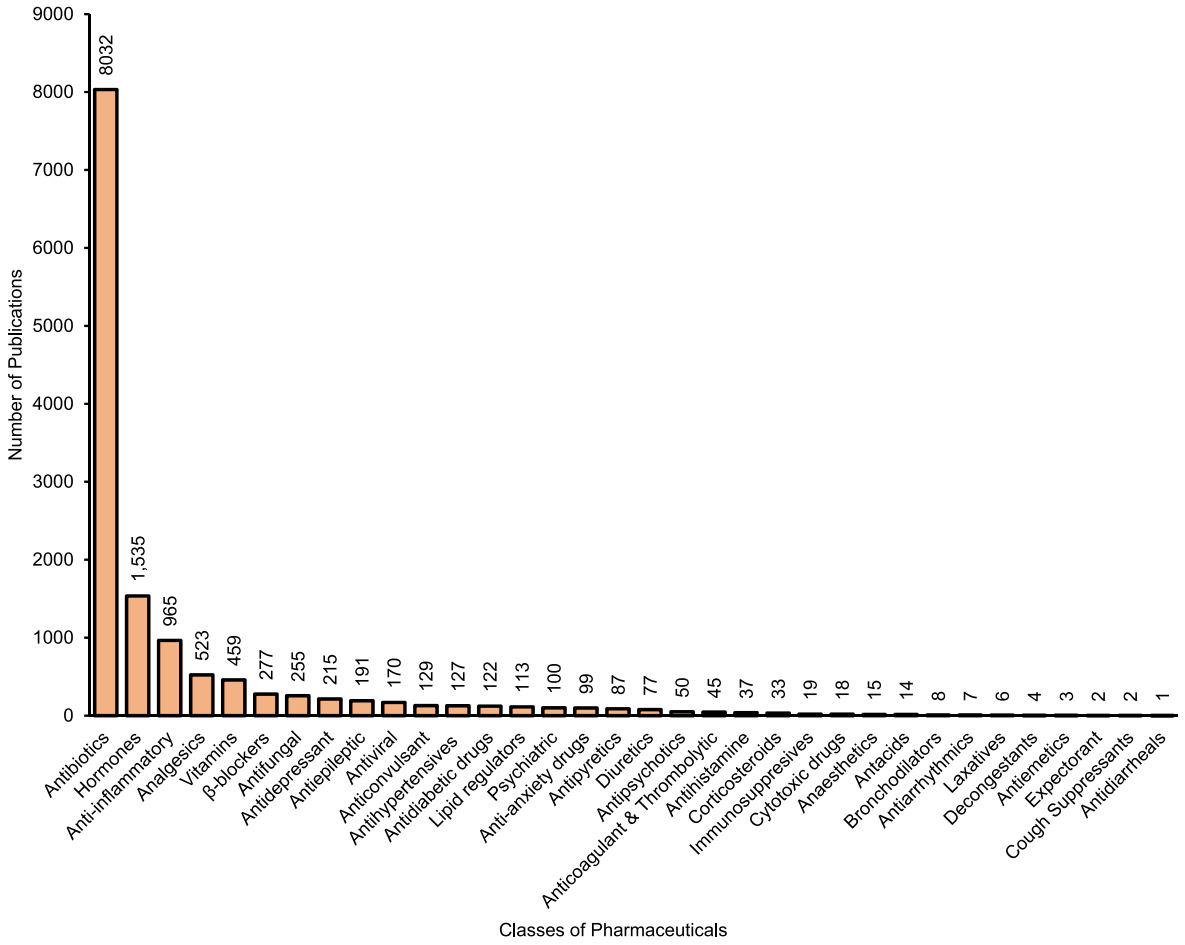

Fig. 2. Publication count for each pharmaceutical class in wastewater.

have toxicity effects, which can cause adverse impacts to aquatic environment (Yuan et al., 2018). Antidepressants(Figure S1, VIII) have received increasing attention since the early 2010s, with a high of close to 60 publications in 2021 showcasing concerns about their occurrence in wastewater. Antidepressant drugs are frequently prescribed to induce sedation and minimize anxiety by suppression of norepinephrine and serotonin reuptake, or by monoamine oxidase (MAO) inhibition (Fernandes et al., 2021; Golbaz et al., 2023).

A sharp peak after 2011 reveals a surge in research attention for β-blocker drugs (Figure S1, VI). In terms of prevalence for β-blockers in wastewater, their potent and chronic effects pose toxicity to aquatic organisms (Patel et al., 2019; Yi et al., 2020). This class could be an important focus for research due to the high concentrations in wastewater, surface water, and drinking water (Khasawneh and Palaniandy, 2021; Ulvi et al., 2022). Antifungal and antiviral APIs have received increased attention more recently, with a sharp uptick in publications since 2019 likely sparked by studies showing adverse consequences in wastewater and ultimately effects on ecosystem processes (Figure S1, VII, X). Antifungal and antiviral drugs have been detected in wastewater, groundwater and surface water because of indiscriminate prophylactic use and improper disposal (Tambosi et al., 2010). In this regard, the opportunistic pathogenic yeast has potency to develop microbial resistance against antifungal and antiviral drugs (Hiller et al., 2019; Monapathi et al., 2021). Therefore, more research into antimicrobial resistance for virus and fungi is warranted.

Other pharmaceutical classes (Figure S1, IX, XI-XIV, XV-XVI) including antiepileptic, anticonvulsant, antihypertensives, antidiabetics, psychiatric drugs and antipyretics have seen a slight increase in research attention over time, though this remains modest. All other classes have less than 10 publications/year over time, and as yet show no clear increasing trend over time (Fig. S1). Singer et al. (2014) examined the distribution, concentrations, and temporal trends of decongestants (xylometazoline, naphazoline, oxymetazoline), antibiotics (ciprofloxacin, cefotaxime, erythromycin, sulfamethoxazole, oxytetracycline), antiemetics (promethazine, cyclizine) and antiviral (oseltamivir) medications in both wastewater and surface water. These pharmaceuticals likely deviate from anticipated environmental concentrations in influent samples due to their limited biodegradability, non-prescription status, and sporadic usage patterns (typically used only as needed). The consumption pattern of these pharmaceuticals has seemingly fluctuated, likely because of poor adherence towards prescribed medications and their wide usage as over-the-counter drugs.

Overall, this analysis provides a broad picture about research intensity over time in terms of publication counts per year. It shows that research on pharmaceutical compounds in wastewater is rapidly increasing, which started with antibiotics, hormones, anti-inflammatory and analgesic pharmaceuticals in the early 2000s, followed in the 2010s by vitamins, antidepressants, and β-blockers, and most recently by research on antifungal and antiviral compounds. These trends indicate which pharmaceutical classes have received more attention over the past decades, which is significant for water quality monitoring, policy regulations and drug development.

5. Available bioassay application in wastewater based on mode of action (MoA)

Pharmaceuticals comprise a diverse array of biologically active APIs tailored to address, prevent, or control a wide spectrum of medical

conditions (Fernandes et al., 2021). This section reviews some of the currently available bioassays and Table 1 provides an overview of the bioassays currently documented in the literature based on prescription volume as enlisted in alphabetical order, with particular emphasis placed on those employed for environmental monitoring in EBM applications, as denoted by the inclusion of an asterisk (*). There are a range of bioassays indicative of cytotoxicity, receptor mediated effects, adaptive stress response, and xenobiotic metabolism (Escher et al., 2021), but not all API classes or MoAs are currently covered by available bioassays. Ideally, a battery of *in vitro* bioassays that cover a range of MoAs and recipient chemicals relevant for the tested water samples is required for comprehensive risk assessment. As all known and unknown chemicals having a similar MoA will contribute to the mixture toxicity in an associated bioassay, the application of a MoA-based test battery can enable a more comprehensive assessment of the toxic potency of a water sample than chemical analysis alone.

Antibiotics play a crucial role in targeting specific components of bacterial cells and can interact synergistically with other medications (Tyers and Wright, 2019; Sullivan et al., 2020). Antibiotics rely on various mechanisms to combat bacterial infections, such as inhibition of cell wall synthesis, hindering protein production, inhibition of nucleic acid synthesis, affecting membrane function, and disrupting metabolic pathways (Soares et al., 2012; Kapoor et al., 2017; Sullivan et al., 2020), as summarised in Table S1. Several assays are commonly applied to test toxicity to bacteria (which includes antibiotic activity) in water, including the Microtox assay and the bacterial luminescence toxicity screen (BLT-Screen) (Tang et al., 2013; Van De Merwe and Leusch, 2015). These assays indicate non-specific toxicity and are usually applied for 15–30 min. These assays detect the effect of many more chemicals than just antibiotics (Escher et al., 2021). However, with the increasing global concerns around antibiotic resistance, there is considerable interest in establishing robust EBM to identify antibiotic contamination in the environment.

The second most widely used group of pharmaceuticals are hormones that affect the function of the endocrine system. These APIs exert their effects mostly through hormone receptor-mediated effects (König et al., 2017; Fuentes and Silveyra, 2019) as their MoAs relates to only agonistic actions on their receptors (Table 1). The endocrine effects of natural and synthetic hormones have been thoroughly assessed in both agonistic and antagonistic modes across a wide range of assays, with particular emphasis on estrogenic, androgenic, progesterone, glucocorticoid, mineralocorticoid, and thyroid activities (Jia et al., 2015; Leusch et al., 2018b). Numerous studies have applied assays indicative of hormone receptor-mediated effects to water samples, particularly estrogenic receptor (ER) as reported in Jia et al. (2015), Leusch et al. (2017), and Escher et al. (2021). Routledge and Sumpter (1996) created a yeast reported gene assay, which has been used extensively to investigate estrogenic activity in wastewater. Estrogenic activity is a particular focus of various bioassays, including the yeast estrogen assay (YES) (Routledge and Sumpter, 1996), a plethora of mammalian reporter gene assays (ERα GeneBLAzer, ERα CALUX, T47D-Kbluc, MELN assay, MVLN assay) and the E-SCREEN breast cancer cell proliferation assay (König et al., 2017; Escher et al., 2021). Many studies have applied these tools to both water samples and individual APIs – for example, Jia et al. (2015) investigated the estrogenic activity of 17α-ethinylestradiol and 17β-estradiol in recycled water using the ER-CALUX and YES assays. König et al. (2017) explored the impact of wastewater effluent discharge in a European river by assessing hormonal activities, including glucocorticoid receptor signalling in agonist and antagonist mode.

For androgenic activity, the yeast androgen screen (YAS) assay and mammalian reporter gene assays (AR-CALUX, AR-GeneBLAzer) are commonly employed for water samples. Leusch et al. (2017) reported that mammalian reporter gene assays for androgenic activity were more sensitive than yeast-based assay. Numerous assays are applied to environmental samples to investigate thyroid activity, including reporter gene assays, the *Xenopus* embryonic thyroid assay (XETA), cell proliferation assays, and other reporter gene assays. Only the whole organism assay (e.g. XETA) detected thyroid activity in water, with no activity detected using the reporter gene assays (Leusch et al., 2018a). König et al. (2017) used PR-GeneBLAzer in surface water, though the effect was masked by cytotoxicity. Both PR CALUX and PR GeneBLAzer assays have applied with some PR activity detected (Leusch et al., 2018b). Furthermore, another study evaluated sensitivity of bioassays in water samples such as GR-GeneBLAzer, GR-Switchgear, and GR-CALUX for glucocorticoid activity (Leusch et al., 2017). The HG5LN-hMR bioassay was applied to detect anti-mineralocorticoid activity in water extracts (Leusch et al., 2018b). Corticosteroids are a type of hormone and an important class of drug as categorised separately by the US Food and Drug Administration list. Under hormones, other hormone receptors such as thyroid, estrogen and androgen receptors were also listed along with glucocorticoid and mineralocorticoid receptors. The corticosteroids contain different MoAs, which are discussed in Table 1, including glucocorticoid receptor agonism, mineralocorticoid activity, and immunosuppression. Corticosteroid drugs such as dexamethasone, prednisolone, aldosterone, act primarily via glucocorticoid receptor agonism, but also affect mineralocorticoid activity (Hodgens and Sharman, 2022). Researchers have extensively discussed the implications and presence of hormones in wastewater by using EBM (Davey and Grossmann, 2016; Robitaille et al., 2022), and there is already a significant body of work (and available EBM tools) for this class of APIs (Escher et al., 2021).

Anti-inflammatory compounds work by effectively inhibiting both forms of cyclooxygenase (COX-1 and COX-2), responsible for generating prostaglandins (PGs) (Ghlichloo and Gerriets, 2019). Nishi et al. (2010) evaluated the potential ecotoxicity of diclofenac, naproxen, ibuprofen, celecoxib and etodolac in wastewater and greywater by determining COX-inhibiting activity. A study conducted by Bernhard et al. (2017) pioneered a cell-based bioassay specifically designed for non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, and successfully applied it to assess anti-inflammatory activity of municipal wastewater. Antipyretic medications share a similar mechanism with NSAIDs, as they both inhibit the COX enzyme, and *in vitro* COX inhibition assays have been used to assess pharmaceutical bioactivity in wastewater samples (Nishi et al., 2010; Inaba et al., 2015).

Analgesics are widely used as painkillers and have specific MoAs: 1) activation of opioid receptors, 2) inhibition of COX enzyme for non-opioid drugs, and 3) blockage of voltage gated ion channels (Table 1). A study conducted by Ohashi and Kohno (2020) emphasized the analgesic impact of acetaminophen, describing its MoA as COX enzyme inhibition. Spetea et al. (2010) examined the opioid pain-relieving potential of morphine, methadone, meperidine, paracetamol, and oxycodone to ascertain G-protein activation via an *in vitro* bioassay employing the μ opioid receptor-mediated mechanism. To date, this assay has not been applied to environmental samples. Petosa et al. (2022) assessed the biological activity of vitamin D in wastewater by using a 10-pathway reporter array and found upregulation of vitamin D receptor in influent and effluent (Table 1). In terms of vitamins, a study conducted by Lundqvist et al. (2023) investigated the interference or disruption of antagonist vitamin D signalling system by *in vitro* bioassays. β-blockers are essential pharmaceuticals for treating cardiovascular disorders. The mechanism of action for β-blockers involves blocking β-adrenergic receptors, including beta-1 (β1), beta-2 (β2), and beta-3 (β3) receptors (Table 1). The β1 adrenergic receptor activity was used to develop a cell-based *in vitro* bioassay, which was applied to explore the toxicological risk of metoprolol in surface water and wastewater (Bernhard et al., 2017).

The MoA for antifungal drugs includes inhibition of ergosterol synthesis, cell membrane disruption, inhibition of nucleic acid production, disruption of fungal cell wall, and interference of fungal metabolism (Table S1). Apart from cell membrane disruption, bioassays for antifungal drugs have been developed based on other modes of action, though none have been applied for water quality monitoring to date.

Table 1
Summary of currently available bioassays with example chemicals.

Classes of Pharmaceuticals	Mode of Action (MoA)	Bioassays	Example Chemicals	References
Anaesthetics	Aesthetic actions of GABAA receptors	–	–	–
	Inhibition of presynaptic voltage-gated channels	–	–	–
Analgesics	Activation of Opioid Receptors	[35S]GTPγS (guanosine-5′-*O*-(3-[35S]thio)-triphosphate) binding assay	14-methoxymetopon, 14-*O*-methyloxymorphone (14-OMO)	Spetea et al. (2010)
	Inhibition of cyclooxygenase (COX) for non-opoid analgesics	In-vitro COX-1/COX-2 Enzyme Inhibition Assay	Celecoxib	Ragab et al. (2017)
	Blockade of Voltage gated ion Channels	Fluorescence Calcium Assays, Fluorescence Membrane Potential Assays	Capsaicin	Chen et al. (2011)
Antacids	Acid neutralization	–	–	–
Antianxiety drugs	Enhancement of GABAergic Inhibition by increasing Gamma-aminobutyric acid (GABA) level	–	–	–
Antiarrhythmics	Voltage gated ion channels modulation	–	–	–
Antibiotics	Inhibition of cell wall synthesis		–	
	Inhibition of protein synthesis	–	–	–
	Inhibition of nucleic acid synthesis	–	–	–
	Disruption of membrane function	–	–	–
	Inhibition of metabolic pathways	Microtox assay[a], BLT screen assay[a], FM-Tox assay	Sulfamethoxazole	(Catterall et al., 2010; Tang et al., 2013; Van De Merwe and Leusch, 2015)
Anticoagulants & Thrombolytics	Inhibition of vitamin K dependent, factor 2a (thrombin) and direct factor 10a	–	–	–
Anticonvulsants	Voltage-gated ion channels modulation,	Calcium imaging assay	4-aminopyridine	Pacico and Mingorance-Le Meur (2014)
	Enhancement of GABAergic Inhibition by increasing Gamma-aminobutyric acid (GABA) level	–	–	–
Antidepressants	Selective Serotonin Reuptake Inhibition (SSRI)	Serotonin (5-HT) Receptor Assays, Dopamine (DA) Receptor Assays, GABA (Gamma-Aminobutyric Acid) Receptor Assays	Vortioxetine, Vilazodone	Ślifirski et al. (2021)
	Serotonin Reuptake inhibition	Serotonin (5-HT) Receptor Assays, Glutamate N-methyl-*d*-aspartate (NMDA) receptor Assays	Vortioxetine, Vilazodone	Ślifirski et al. (2021)
	Monoamine Oxidase (MAO) Inhibition	[a]Monoamine transporter inhibition (MAT) assay	Vortioxetine, Vilazodone, Paroxetine, Sertraline, Venlafaxine, Tramadol	Zhang et al. (2023)
	Norepinephrine Reuptake Inhibition (Tricyclic antidepressants-TCA)	3H-Nisoxetine Binding Assay	Nisoxetine	Zhao et al. (2009)
	Serotonin and Norepinephrine Reuptake Inhibition (SNRI),	3H-Citalopram Binding Assay	Citalopram	Zhao et al. (2009)
Antidiabetic drugs	Activation of the K-ATP channel-dependent pathway	–	–	–
Antidiarrheals	Opioids agonist	Cell-Based Assays for Inflammation, antibacterial activity assay	Nitazoxanide	Ahmed et al. (2021)
	Adsorption	–	–	–
	Intestinal fluid modification	–	–	–
Antiemetics	Serotonin (5-HT3) receptor antagonists	[35S]-GTPγS ([35S]-guanosine-5'-*O*-(3-thio)-triphosphate) assay	–	Pottie and Stove (2022)
	Neurokinin-1 (NK1) receptor antagonists	–	–	–
	H1 Receptor Antagonists	–	–	–
	Acetylcholine (M) Receptor Antagonists	–	–	–
	Synthetic Cannabinoids	–	–	–
	Corticosteroids	[a]GR-GeneBLAzer, [a]GR-CALUX, [a]GR-Switchgear, [a]CV-1 GR assay	Dexamethasone, Prednisolone	König et al. (2017)
Antiepileptics	Voltage-gated ion channels modulation	Calcium imaging assay	4-aminopyridine	Pacico and Mingorance-Le Meur (2014)
	Enhancement of GABAergic Inhibition by increasing Gamma-aminobutyric acid (GABA) level	–	–	–
Antifungals	Inhibition of Ergosterol biosynthesis	Antifungal disk diffusion assay, biofilm assay, MTT assay	Fluconazole, Voriconazole, Posaconazole, Itraconazole, Amphotericin B, Caspofungin	(Tebbets et al., 2012; Kryczyk et al., 2017; Berkow et al., 2020)
	Cell Membrane Disruption	Bioluminescence assay, microdilution assay	Amphotericin B, Voriconazole	(Galiger et al., 2013; Balouiri et al., 2016)

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Table 1 (*continued*)

Classes of Pharmaceuticals	Mode of Action (MoA)	Bioassays	Example Chemicals	References
	Inhibition of Nucleic Acid Synthesis	Antifungal disk diffusion assay	2-nitro-5-(4-nitroStyryl) furan	Tebbets et al. (2012)
	Disruption of Fungal Cell Wall	Cell viability assay, Bioluminescence assay	Anidulafungin	(Jayaraman et al., 2008; Galiger et al., 2013)
	Interference with Fungal Metabolism	Hik1 Saccharomyces bioassay, Biofilm assay, MTT assay, antifungal microdilution assay	2-(methylthio)-5-[(5-nitro-1,3-thiazol-2-yl)thio]-1,3,4-thiadiazole, Fluconazole, Amphotericin B	(Tebbets et al., 2012, 2013; Pacholak et al., 2022)
Antihistamines	H1 Receptor Antagonism, Inhibition of Allergic Symptoms	Histamine Receptor Binding Assays (H1, H2, H3 receptors), IgE-Mediated Allergic Response Assays, [a]TGFα Shedding Assay	Fexofenadine, Trazodone, Amitriptyline	(Schnyder and Pichler, 2009; Ihara et al., 2015; Mehta et al., 2021)
Antihypertensives	Angiotensin II receptor blockers	–	–	–
Anti-inflammatory	Inhibition of cyclooxygenase (COX-1)	[a]COX (ovine) screening assay, [a]NSAID biosensor cell line expressed fluorescent redox sensor roGFP3 together with the human cyclooxigenase-1	Diclofenac, Naproxen	(Nishi et al., 2010; Bernhard et al., 2017; Simon et al., 2022)
	Inhibition of cyclooxygenase (COX-2)	In-vitro COX-1/COX-2 Enzyme Inhibition Assay	Celecoxib	Ragab et al. (2017)
Antipsychotics	Dopamine D2 receptor antagonism	Receptor binding assays (serotonin, dopamine, adrenergic receptors), [a]TGFα Shedding Assay	Chlorpromazine, Promazine	(Siafis et al., 2018; Zhang et al., 2018)
	Serotonin 5-HT2A receptor antagonism	[a]TGFα Shedding Assay	Chlorpromazine, Promazine	Zhang et al. (2018)
	Partial agonism or modulation of glutamate receptors	–	–	–
Antipyretics	Inhibition of cyclooxygenase (COX 1/2/3) producing prostaglandins (PGs)	[a]COX (ovine) screening assay, [a]NSAID biosensor cell line expressed fluorescent redox sensor roGFP3 together with the human cyclooxigenase-1	Diclofenac sodium, Celecoxib	(Nishi et al., 2010; Simon et al., 2022)
Antivirals	Nucleoside analog inhibition of nucleic acid synthesis, viricidal	Cell-cell fusion assay, Immunoprecipitation assay	Acyclovir, Entecavir, Sofosbuvir, Cidofovir	(Herschhorn et al., 2011; Rumlová and Ruml, 2018)
	Non-nucleoside noncompetitive inhibition	Plaque reduction assay, Enzyme linked Immunosorbent assay (ELISA), Fusion inhibition assay, PCR based assay	Nevirapine, Efavirenz, Delavirdine	Rumlová and Ruml (2018)
	Inhibit escape of virus from endosomes	Fluorescence-linked immunosorbent assay (FLISA)	Remdesivir, Amantadine, Rimantadine	Rumlová and Ruml (2018)
	Inhibit neuraminidase and Viral adsorption	CisBio Bioassays, Fast Assembly Inhibitor Test for HIV (FAITH) assay	Acyclovir, Lopinavir, Oseltamivir	Rumlová and Ruml (2018)
	Inhibition of viral entry	Core-stability assay, fate of capsid assay	Chloroquine, Paroxetine	Rumlová and Ruml (2018)
	Inhibition of protease and integrase	Plaque reduction assay, Enzyme linked Immunosorbent assay (ELISA), Fusion inhibition assay,	Ritonavir	Rumlová and Ruml (2018)
	Inhibition of membrane fusion	Fusion inhibition assay	Enfuvirtide	Rumlová and Ruml (2018)
Bronchodilators	Inhibition of beta-2 receptor (G-protein coupled receptor)	Cyclic adenosine monophosphate (cAMP) assay	Salbutamol	Muddle et al. (2020)
Corticosteroids	Glucocorticoid receptor agonism	[a]GR-GeneBLAzer, [a]GR-CALUX, [a]GR-Switchgear, [a]CV-1 GR assay	Dexamethasone, Prednisolone	König et al. (2017)
	Mineralocorticoid activity	[a] HG5LN hMR	Aldosterone	(Leusch et al., 2018b; Neale et al., 2020b)
	Immunosuppression	–	–	–
Cough Suppressants	Affected by Pharmacokinetic activity towards bronchial secretions, digesting enzymes	–	–	–
Cytotoxic drugs	Inhibition of cell division and cell growth (Anticancer Drugs)	[a]Various assays for cytotoxicity, such as MTT/MTS, resazurin, neutral red uptake etc.	Doxorubicin, Methotrexate	(Leusch et al., 2014; Parrella et al., 2014; Escher et al., 2021)
Decongestants	Activation of postjunctional a-adrenergic receptors	Alpha-Adrenergic Receptor Binding Assays	–	Corboz et al. (2007)
Diuretics	(Thiazide) inhibiting the sodium-chloride symporter	–	–	–
	Inhibition of NKCC2 transporter	–	–	–
	Aldosterone Antagonism,	–	–	–
	Epithelial sodium channels (ENaC) inhibition,	–	–	–
	Carbonic Anhydrase Inhibition	–	–	–
Expectorant	Increasing volume of secretions and reduction of viscosity in trachea	–	–	–
Hormones	Estrogen receptor signalling	[a]YES, [a]ERα CALUX, [a]ERα GeneBLAzer, [a]MELN, [a]HeLa-9903, [a]MVLN, [a]T47D-KBluc, [a]E-SCREEN	17β-Estradiol, Estriol, Estrone, 17α-Ethinylestradiol	(Routledge and Sumpter, 1996; Coldham et al., 1997; Leusch et al., 2010; Jia et al., 2015; Escher et al., 2021)
	Androgen receptor	[a]AR-CALUX, [a]YAS, [a]AR GeneBLAzer, [a]MDA-kB2	Dihydrotestosterone, Testosterone, Trenbolone	(Leusch et al., 2014; Escher et al., 2021)

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Table 1 (*continued*)

Classes of Pharmaceuticals	Mode of Action (MoA)	Bioassays	Example Chemicals	References
	Progesterone receptor	[a]PR-CALUX, [a]PR GeneBLAzer	Levonorgestrel	(Jia et al., 2015; König et al., 2017)
	Glucocorticoid receptor	[a]GR-GeneBLAzer, [a]GR-CALUX, [a]GR-Switchgear, [a]CV-1 GR assay	Dexamethasone, Prednisolone	König et al. (2017)
	Mineralocorticoid receptor	[a]HG5LN MR	Aldosterone	(Leusch et al., 2018b; Escher et al., 2021)
	Thyroid receptor	[a]T-SCREEN, [a]TRβ-CALUX, [a]TRβ-GeneBLAzer, [a]GH3.TRE-Luc, [a]Xenopus Embryonic Thyroid Assay (XETA), [a]PC-DR-LUC	Triiodothyronine(T3), Thyroxine (T4)	(Leusch et al., 2014, 2018b)
Immunosuppressive	Inhibition of inflammation, interference in cell proliferation and immune responses towards antigen	–	–	–
Laxatives	Bulk forming substances by retaining fluid, 5-Hydroxytryptamine receptor agonism, Guanylate Cyclase Agonism	–	–	–
Lipid regulators	HMG-CoA Reductase Inhibition (Statins)	HMG-CoA reductase inhibition assay, [a]HG5LN hPXR assay	Pravastatin, Mevastatin	(Creusot et al., 2010; Abeysekera et al., 2022)
	Inhibition of Cholesterol Absorption (Fibrates)	Cholesterol micellization inhibition Assays, Cholesterol esterase inhibition assay	Fenofibrate, Bezafibrate	Abeysekera et al. (2022)
	Activation of Peroxisome Proliferator-Activated Receptors (PPARs)	Peroxisome Proliferator-Activated Receptor (PPAR) Assays	Rosiglitazone	Jia et al. (2015)
	Inhibition of Lipoprotein Lipase (LPL)	Low-density lipoprotein (LDL) Receptor Assays	Mevastatin, Pravastatin	Abeysekera et al. (2017)
	Bile Acid Sequestration,	Lipid metabolism assays	Colesevelam	Abeysekera et al. (2017)
Psychiatrics	Antidepressants (SSRI, TCAs, MAOs)	[a]Monoamine transporter inhibition (MAT) assay	Vortioxetine, Vilazodone, Paroxetine, Sertraline, Venlafaxine, Tramadol	Zhang et al. (2023)
	Antianxiety medications (GABA) inhibition	–	–	–
	Blockage of serotonin and dopamine receptors	[a]TGFα Shedding Assay	Chlorpromazine, Promazine	Zhang et al. (2018)
	Reduction in neurotransmitter level	–	–	–
Vitamins	Receptor signalling of fat-soluble vitamin K, D, E, A	[a]10-Pathway Reporter Array for vitamin D receptor	1α,25-dihydroxyvitamin D3	Petosa et al. (2022)
	Water soluble vitamins (B1, B2, B3, B5, B6,B9, B12)	–	–	–
β-blockers	β-adrenergic Receptor Blockade e.g., beta-1 (β1), beta-2 (β2), and beta-3 (β3) receptors	[a]Beta-blocker biosensor cell line (CEPAC cAMP FRET sensor having Pstz-4 vector with tetracycline promotor), [a]transforming growth factor-α (TGFα) shedding assay	Isoproterenol, Metoprolol, Atenolol	(Rastogi et al., 2015; Bernhard et al., 2017; Zhang et al., 2020)

[a] Application of bioassays in environmental monitoring to detect pharmaceuticals, such as synthetic hormones, anti-inflammatory, β-blockers, antidepressants, psychiatric drugs, antihistamines, antipsychotics, corticosteroids, antipyretics, antiarrhythmics, and antiemetic drugs. The dotted line (—) represents no data found in the literature.

Antidepressant medications function by increasing neurotransmitter levels, acting via MoA that promote the stimulation of postsynaptic receptors and facilitate neurotransmission (Sheffler et al., 2019). Examples include selective serotonin reuptake inhibition (SSRI), monoamine oxidase (MAO) inhibition, norepinephrine reuptake inhibition (tricyclic antidepressants), and serotonin & norepinephrine reuptake inhibition (SNRI) (Table S1). Ihara et al. (2021) developed a monoamine transporter (MAT) inhibition assay to detect the biological activity of both antidepressants, such as fluoxetine, sertraline and citalopram, and wastewater extracts from Japan. The assay was also applied to predict antidepressant equivalent concentrations in wastewater effluent from Japan and England to identify which antidepressants were contributing the most to the overall activity (Zhang et al., 2023).

Antiepileptic and anticonvulsant medications share similar MoAs, which encompasses the modulation of voltage-gated ion channels and the activation of GABAergic inhibition by elevating levels of Gamma-Aminobutyric Acid (GABA). Antihypertensives, such as candesartan, telmisartan, losartan, eprosartan, and irbesartan, are used to manage cardiovascular conditions (Table S1) and work by blocking the angiotensin II receptor (Khalil and Zeltser, 2022). Antiviral drugs are primarily employed in the treatment of viral infections. These medications target viruses through various mechanisms (Kausar et al., 2021). Diverse MoAs encompass inhibiting nucleic acid synthesis, preventing virus escape from endosomes, employing non-nucleoside non-competitive inhibition, blocking viral adsorption and neuraminidase, inhibiting viral entry, interfering with protease and integrase activation, and inhibiting membrane fusion (Kausar et al., 2021). Antiviral drugs also strengthen the cell's resistance to viruses, similar to interferons. These endpoints have been used to determine the ecotoxicological impacts of antiviral drugs and determine antiviral activity, using *in vitro* assays such as the cell-virus-fusion assay, cell-cell fusion assay, and biochemical assay, but have not yet been applied in water quality monitoring (Rumlová and Ruml, 2018) (Table 1).

Lipid regulators act via the inhibition of HMG-CoA reductase activity (statins), interference with cholesterol absorption (fibrates), activation of peroxisome proliferator-activated receptors (PPARs), inhibition of the lipoprotein lipase enzyme, and bile acid sequestration. Statin drugs, such as atorvastatin, fluvastatin, rosuvastatin, pitavastatin and pravastatin are widely used to reduce cholesterol levels (Feingold, 2016). Abeysekera et al. (2022) determined bioactivities for lipid regulators using *in vitro* bioassays such as cholesterol micellization inhibition assays, cholesterol esterase inhibition assay, low-density lipoprotein (LDL)

receptor assays, none of which have yet been applied for water monitoring. For lipid regulators, the G5LN hPXR assay has been applied for environmental monitoring, but PXR is a highly promiscuous receptor, so will detect a lot more types of chemical contaminants than just statins.

Psychiatric pharmaceuticals include antianxiety medications, sedatives, and anxiolytics, which have various mechanisms, such as inhibition of GABA levels, neurotransmitter levels, and blockage of serotonin and dopamine receptors (i.e., chlorpromazine, promazine). Some psychiatric drugs, such as vortioxetine, vilazodone, paroxetine, sertraline, venlafaxine, and tramadol, have similar MoAs as antidepressants with their MoAs in psychiatric drugs targeted for assay development (Table 1). Moreover, antipsychotic drugs work as antagonist for dopamine D2 receptor, serotonin 5-HT2A receptor and partial agonist for glutamate receptor. Zhang et al. (2018) assessed the biological activity of antipsychotic compounds in wastewater samples by employing a transforming growth factor (TGFα) assay to evaluate their antagonistic effects on the serotonin 5-HT2A receptor. Antidiabetic medications are employed to stimulate potassium channels through an adenosine triphosphate (ATP)-dependent pathway in the treatment of diabetes (Table S1). However, no bioassay have been developed to date to assess antidiabetic pharmaceuticals using this particular MoA (Rehani et al., 2019). Parrella et al. (2014) assessed the biological activity of cytotoxic drugs such as doxorubicin in water systems by MTT ((3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay that contribute to evaluate the cytostatic environmental risk. Ideally, when applying cell-based bioassays for water quality monitoring, cytotoxicity should always be measured in parallel with specific endpoints to make sure not to interfering with the assay (Leusch et al., 2014; Escher et al., 2021). There are many different assays to measure cytotoxicity, including resazurin, lactate dehydrogenase (LDH), neutral red uptake, MTT or tetrazolium assay (Escher et al., 2021). Concurrent cytotoxicity can potentially lead to false-negative or false-positive effects. As all chemicals can potentially cause cytotoxicity at sufficiently high concentrations, cytotoxicity assays are non-specific and cannot be used to identify certain pharmaceuticals (Escher et al., 2021). In addition, some pharmaceutical classes can trigger several adverse outcome pathways (AOPs), and it is not necessarily straightforward to link a particular chemical class to a specific biological effect. Cytostatic drugs are another class of APIs used in cancer therapy and induce DNA damage causing cytotoxic, mutagenic, genotoxic or carcinogenic impacts in non-targeted organisms.

Antiarrhythmics such as amiodarone primarily function by modulating voltage-gated ion channels. Decongestants are implicated in inducing physiological effects and activating postjunctional α-adrenergic receptors. Corboz et al. (2007) investigated the impacts of α2-adrenoceptor agonists BHT-920 and PGE-6201204 acting as decongestants by using nasal mucosa contractile bioassays useful for the treatment of nasal congestion. This assay has never been used in environmental monitoring and may therefore represent an opportunity to adapt bioassay of decongestants for future research. Antiemetics employ various mechanisms to alleviate nausea and vomiting, targeting specific chemoreceptor pathways. These actions encompass serotonin (5-HT3) receptor antagonism, neurokinin-1 (NK1) receptor antagonists, histamine H1 receptor antagonists, acetylcholine (M) receptor antagonists, and corticosteroids (Hauser et al., 2018). Pottie and Stove (2022) highlighted the characterisation of [35S]-GTPγS ([35S]-guanosine-5'-*O*-(3-thio)-triphosphate) assay for serotonin receptor 5-HT2AR but it has never been applied to environmental samples.

Antianxiety drugs enhance GABAnergic Inhibition by increasing Gamma-aminobutyric acid (GABA) levels. There is no bioassay available for antianxiety drugs that has been validated for environmental monitoring (Table 1). Antidiarrheal medications predominantly consist of opioid derivatives employed in the management of diarrhea. These pharmaceuticals act as opioid-receptor agonists, facilitating the absorption of fluids and bringing about alterations in intestinal fluid dynamics (Schiller, 2017; Paul, 2021). An antibacterial activity assay for opioid agonist (i.e., Nitazoxanide) exists but has not applied in water monitoring (Ahmed et al., 2021) as reported in Table 1. Relevant MoAs for the remaining pharmaceutical classes (antihypertensive, anticoagulants & thrombolytics, diuretics, bronchodilators, antacids, laxatives, anaesthetics, expectorant, cough suppressants and immunosuppressive compounds) are presented in Table S1 and Table 1, but bioassays relevant to an EBM context could not be identified.

To determine whether other MoA might be identified by considering the bioactivity of specific compounds, we searched the list of representative compounds in Table S3 in the United States Environmental Protection Agency (USEPA) computational toxicity dashboard (CompTox, 2023). This online tool facilitates the provision of information about environmental fate, usage, exposure, toxicity data, and responses to *in vitro* bioassays for thousands of chemicals. ToxCast confirmed the assays found in the literature for specific MoA, and sometimes also identified effects associated with side-effects of specific APIs instead of the therapeutic MoA. Table 1 provides a summary of the bioassays identified through the literature search. Table S3 indicates that in addition to the Tox21 assays for estrogen receptor signalling, there are also other assays in CompTox that can detect estrogen and other hormone receptors (e.g., Novascreen (NVS)). Bioassays for NSAIDs were NVS ENZ_oCOX1 and NVS ENZ_oCOX2, which were active for diclofenac sodium and celecoxib, respectively.

EBM involve the detection of both the parent compounds and their transformation products in various environmental samples, including wastewater, surface water, and drinking water (Patel et al., 2019; Mutuku et al., 2022). This section identified current knowledge gaps in bioassay availability, which will be discussed in Section 6.

6. Opportunities for development of new bioassays for water quality monitoring

This section aims to identify existing opportunities in bioassay application based on MoAs that have not been incorporated into EBM yet and to explore the potential for developing new bioassays to further increase the coverage of EBM for water quality monitoring. There are several potential avenues for the development of bioassays targeting these specific MoAs or for modifying existing bioassays rooted in these mechanisms. Importantly, these approaches have not been employed in an EBM context, as outlined in Table 1. For example, current bioassays for inhibition of metabolic pathways such as the Microtox assay and BLT-Screen will detect both antibiotic and non-antibiotic pharmaceuticals (Tang et al., 2013; Neale et al., 2017). They respond to most chemicals, including antibiotics, after a typical exposure period of 15–30 min, which represents non-specific toxicity (Tang et al., 2013; Neale et al., 2017). Previous studies have observed increased sensitivity to antibiotic effects over time (Backhaus et al., 1997; Zou et al., 2012), so increasing the exposure duration of these assays may make them more suitable for monitoring antibiotics. In this regard, several other endpoints associated with antibiotics have not been explored and may represent novel approaches that can be adopted for EBM (Table 1).

Anti-inflammatory drugs function by inhibiting both COX-1 and COX-2 enzymes. Those with the COX-1 form are linked to certain bioassays, such as the COX (ovine) screening assay and the NSAID biosensor cell line expressing fluorescent redox sensor roGFP3 alongside human cyclooxygenase-1. These assays have already been applied (to a limited extent) towards environmental monitoring. On the other hand, the COX-2 form of anti-inflammatory drugs involves a COX-2 inhibition assay, which has not been utilized for environmental samples. Likewise, analgesics with diverse MoAs are associated with various bioassays, including [35S] GTPγS binding assay, *in vitro* COX-1/COX-2 enzyme inhibition assay, fluorescence calcium assays, and fluorescence membrane potential assays. The COX inhibition assay exhibits reduced specificity, potentially responding to both anti-inflammatory drugs and analgesics. Specific bioassays have been developed to test antifungal APIs, such as the Hik1 Saccharomyces bioassay, antifungal disk diffusion

assay, biofilm assay, and cell viability assay (refer to Table 1). Notably, these bioassays associated with the different MoAs of antifungal drugs have not yet been employed for environmental samples. The calcium imaging assay has proven to be effective in evaluating antiepileptics, anticonvulsants, and antihypertensives in clinical settings and may present a valuable opportunity for repurposing an existing bioassay towards EBM.

Opportunities also exist where analogous MoAs of API classes could be applied. For example, the calcium imaging assay has been employed to study antiarrhythmics that impact voltage-gated ion channels, suggesting the potential for exploring a comparable assay in the context of anticonvulsants. Antidepressants have multiple endpoints detected by *in vitro* bioassays, which include Serotonin (5-HT) Receptor Assays, Dopamine (DA) Receptor Assays, GABA (Gamma-Aminobutyric Acid) Receptor Assays for SSRI, and serotonin reuptake inhibition, but these have not yet been applied to environmental samples. Some API classes such as antidepressants, antiepileptic, anticonvulsant, psychiatric, and antianxiety drugs target GABA inhibition. Exploring assay development to target GABAergic inhibition would increase the scope for EBM to cover numerous API contaminants in water. Antiviral drugs face a similar concern, with assays developed for several MoAs (e.g., cell-cell fusion assay, immunoprecipitation assay, plaque reduction assay, enzyme linked immunosorbent assay (ELISA), PCR-based bioassay, fluorescence-linked immunosorbent assay (FLISA), core-stability assay, and fate of capsid assay), but their applicability towards environmental monitoring has not been established. Considering the current focus on antivirals and the high and increasing production volumes for this class of APIs, there is a clear gap in the application of relevant bioassays for environmental monitoring of antiviral drugs.

There is a robust suite of bioassays for lipid regulators based on their MoAs such as cholesterol micellization inhibition assays, cholesterol esterase inhibition assay, low-density lipoprotein (LDL) receptor assays (Abeysekera et al., 2017) but these assays are not used for water monitoring. Currently, no bioassays are available for diuretics, anticoagulant & thrombolytics, immunosuppressive, antiarrhythmics, expectorants, cough suppressants, laxatives, antihypertensives, anaesthetics, antidiabetics, antianxiety, and antacid drugs applied in water samples (Table 1). These gaps highlight key areas where future research can make valuable contributions to bioassay development for environmental monitoring. Consequently, development of new bioassays or adopting existing bioassays towards environmental samples is critical to detect a broader range of commonly used pharmaceutical compounds and would improve the ability of EBM to quantify important APIs in the environment. Considering the availability of assays focusing on multiple or similar MoAs and taking into account the consumption volume (kg/year), the following priority list for APIs is recommended, including antifungal drugs, antiviral drugs, analgesics, antianxiety drugs, anticonvulsants, and diuretics. Overall, considering the MoAs of pharmaceutical classes can aid in the development of innovative bioassays or the exploration of novel applications for existing bioassays for environmental monitoring of APIs.

7. Conclusions

Antibiotics have received the highest attention from researchers among the 34 pharmaceutical classes due to their considerable annual production and occurrence in wastewater and the environment. When considering their MoAs, the adoption of *in vitro* bioassay methods for monitoring pharmaceuticals in aquatic environments holds immense promise. Synthetic endocrine hormones have been well-addressed using *in vitro* bioassays, while assays targeting NSAIDs, beta blockers, monoamine transporter inhibitors and serotonin 5-HT2A inhibitors have also been applied to water samples to detect APIs. However, while promising bioassays exist for analgesics, antiviral, antifungal, and antiepileptic pharmaceuticals, they have not yet been widely applied to water samples. Several pharmaceutical classes such as antibiotics, antihypertensives, antidiabetic drugs, diuretics, anaesthetics, and laxatives lack comprehensive *in vitro* bioassays for detecting the relevant endpoints. Therefore, there is an urgent need to develop a new series of bioassays and to utilise the existing bioassays applied for water monitoring that can detect a wider range of commonly used pharmaceutical compounds. Such an advance would be beneficial in enhancing the capabilities of EBM to detect and quantify APIs, particularly when applied to environmental samples. Promoting the application of EBM could provide significant confidence for policymakers, regulatory bodies, and water quality managers through generating a framework for effect-based trigger value (EBT) exceedances and maximizing the experience towards systematic EBM approaches in a regulatory context, and in water quality and surveillance monitoring. This multifaceted approach notably enhances the effectiveness of environmental risk assessment for pharmaceutical contamination in water.

CRediT authorship contribution statement

Rameesha Tanveer: Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Peta A. Neale:** Writing – review & editing, Visualization, Supervision. **Steven D. Melvin:** Writing – review & editing, Visualization, Supervision. **Frederic D.L. Leusch:** Writing – review & editing, Visualization, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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